New Approaches for Chemosensitization of Refractory Leukemia by Modulation of Programmed Cell Death

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von

Laura Bonapace

aus

Italien

Promotionskomitee

Prof. Michael O. Hengartner (Vorsitz)

Prof. Josef Jiricny

Prof. Jean-Pierre Bourquin (Leitung der Dissertation)

PhD Beat Bornhauser

Prof. Beat Schäfer

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Zurich, July 2010
Laura Bonapace

To my family

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Summary

Acute lymphoblastic Leukemia (ALL) is a disease of the bone marrow and the blood, which represents the most recurrent cancer in childhood. ALL can be cured with multimodal chemotherapy in approximately 80% of the cases. Short and long term toxicity can be significant. Nevertheless a substantial number of patients with ALL will eventually relapse by acquiring treatment resistance. Therefore this disease is still one of the leading causes of death in children. On current treatment protocols of the BFM study group (one of the largest cooperative study groups for the treatment of ALL, still named Berlin-Frankfurt-Muenster (BFM), to credit the important contribution of the founding centers) for childhood ALL, a group of patients with exceedingly poor prognosis can be identified based on the persistence of minimal residual disease. Most of these very high risk patients (VHR-ALL) are also resistant to glucocorticoids (GC) and will qualify for experimental therapy upfront. A general model in the field proposes the blockade of the intrinsic apoptotic pathway as a mechanism of GC resistance. Gene expression profiling studies strongly suggest that GC-resistance is associated with upregulation of metabolic signatures (glycolysis, oxidative phosphorylation, cholesterol biosynthesis and glutamate metabolism) and activation of PI3K/AKT/mTOR pathways [1]. Consistent with these data, we demonstrated that interference with AKT signaling using arsenic trioxide can restore sensitivity to glucocorticoids in ALL cells from prednisone poor responders patients (publication1) [2]. This work identified members of the pro-survival PI3K/AKT pathway as rational targets for therapeutic intervention in this subgroup of high risk ALL. Armstrong et al [1] proposed based on gene expression profiling, that overexpression of the anti-apoptotic protein MCL-1 is associated with resistance to glucocorticoids in ALL. We therefore decided to evaluate the new compound obatoclax, a pan-BCL-2 inhibitor selected based on its capability to bind and antagonize MCL-1, to act as a chemosensitizer. Using a xenograft model in immunodeficient mice, we could demonstrate

that obatoclax conveys a strong and broad chemosensitization effect in primary ALL cells from patients with highly resistant disease (publication 2) [3]. Unexpectedly, re-sensitization to glucocorticoids resulted from activation of an alternative cell death pathway to bypass the apoptotic blockade in GC resistant cells. This effect was dependent on rapid and specific induction of autophagy, as demonstrated in rescue experiments using pharmacologic inhibitors of autophagy or specific knock-down of autophagy genes. However, execution of cell death was also dependent on RIP-1 kinase activity, which we show to act downstream of autophagy. Furthermore, combination of obatoclax and dexamethasone resulted in characteristic cell death morphology by electron microscopy that is reminiscent of induction of a necrotic cell death pathway [4], also referred to as necroptosis [5-6]. Importantly, in combination with other non-glucocorticoid antileukemic agents, obatoclax also conveyed very effective chemosensitization, but this effect was associated with induction of mitochondrial apoptosis. The specific induction of autophagy dependent necroptosis in combination with dexamethasone was associated with two events that could contribute to the cell death trigger mechanism. First, low concentrations of obatoclax were sufficient to disrupt the interaction between the autophagy regulator Beclin-1 with the anti-apoptotic regulator MCL-1 which may interfere with repression of autophagy by this BCL-2 family member. Second, only in combination with dexamethasone, obatoclax resulted in strong inhibition of mTOR kinase activity, which is known to modulate induction of autophagy. Finally, we could show strong preclinical in vivo activity for this approach, using a xenograft model with relevant patient samples. Taken together, this work provides compelling evidence to support a rapid translation of this approach to the clinics. Accordingly, a phase-I clinical trial is in final preparation in collaboration with major European Institutions. Our data provide the basis for the evaluation of the biological response to the combination of obatoclax and dexamethasone in patients with refractory ALL and have been critical to obtain first priority

for this clinical study in Europe. From a mechanistic standpoint, our data provide a unique experimental system to investigate the molecular events that link autophagy, necroptosis and apoptosis in a clinically highly relevant setting. Finally, our experimental platform can be expanded for high content screens, which will open new avenues for the investigation of drug resistance in ALL and the triage of the most effective therapeutic agents for highly resistant disease.

Zusammenfassung

Akute lymphoblastische Leukämie (ALL) ist eine Krankheit des Knochenmarkes und Blutes und ist die häufigste Krebsart bei Kindern. ALL kann mit multimodaler Chemotherapie in zirka 80% aller Fälle geheilt werden, wobei aber Kurzzeit- und Langzeittoxizität signifikant sein können. Leider gelingt es nicht, die restlichen 20% zu heilen, da sie auf die Chemotherapie resistent sind. Deshalb ist die ALL immer noch eines der grössten Probleme in der Pädiatrie. Derzeitige Behandlungsprotokolle der BFM-Studiengruppe (eine der grössten kooperativen Studiengruppen für die Behandlung von ALL, benannt nach den Gründungszentren Berlin-Frankfurt-Münster) identifizieren basierend auf anhaltender minimaler Resterkrankung (MRD) eine Patientengruppe mit ausgesprochen schlechter Prognose. Die meisten dieser Hochrisikopatienten (VHR-ALL) sind auch auf Glukokortikoide (GC) resistent und qualifizieren für experimentelle Therapie. Als gängiges Modell für den Mechanismus der GC-Resistenz wird die Blockade des intrinsischen apoptotischen Pfades gehandelt. Genexpressionsprofile deuten daraufhin, dass die GC-Resistenz mit der Hochregulation von metabolischen Signaturen (Glykolyse, oxidative Phosphorylierung, Cholesterinbiosynthese und Glutamatmetabolismus) und der Aktivierung des PI3K-AKT-mTOR Signalweges zusammenhängt [1]. In Übereinstimmung mit diesen Daten haben wir gezeigt, dass durch die Inhibition des AKT-Signalweges mit Arsentrioxid Patienten mit GC-Resistenz resensitisiert werden (Publikation 1) [2]. In der Untergruppe der Hochrisikopatienten identifiziert diese Arbeit Mitglieder des PI3K-AKT Signalweges als therapeutische Zielproteine. Armstronget al. [1] schlugen aufgrund von Genexpressionsprofilen vor, dass die Überexpression des anti-apoptotischen Proteins MCL-1 mit der GC-Resistenz in der ALL zusammenhängt. Daher haben wir uns entschlossen, den neuen Wirkstoff Obatoclax, einen pan-BCL-2-Inhibitor, zu evaluieren. Dieser Inhibitor wurde mit der Hoffnung selektionert, aufgrund seiner MCL-1 antagonisieren der Eigenschaft

glukokortikoid-resistente ALL Zellen auf ebendiese Glukokortikoide wieder empfindlich zu machen. Mit Hilfe unseres Xenograft Modells mit immundefizienten Mäusen konnten wir zeigen, dass Obatoclax einen starken und breitbandigen Chemosensitisierungseffekt in primären ALL-Zellen von VHR-ALL-Patienten hat (Publikation 2) [3]. Unerwarteterweise erfolgte die Resensitisierung auf Glukokortikoide durch die Aktivierung eines alternativen Zelltodweges und umging die Blockade der Apoptose in GC-resistenten Zellen. Dieser Effekt war abhängig von der schnellen und spezifischen Induktion der Autophagie und wurde durch pharmakologische Inhibition der Autophagie oder spezifische Inaktivierung von Autophagiegenen mittels RNA Interferenz aufgehoben. Zudem war die Ausführung des Zelltodes auch abhängig von der Aktivität der RIP-1 Kinase, welche dem Autophagie-Signalweg nachgeschaltet ist. Darüber hinaus zeigten elektronenmikroskopische Aufnahmen, dass Zellen nach der Behandlung mit der Kombination von Obatoclax und dem Glukokortikoid Dexamethason eine charakteristische Morphologie des Zelltodes aufweisen, welche der Nekrose ähnlich ist [4] und auch Nekroptose genannt wird [5-6]. Es ist wichtig zu erwähnen, dass Obatoclax resistente Zellen auch auf andere, nicht-glukokortikoidale antileukämische Medikamente empfindlich gemacht hat. Dieser Effekt war aber abhängig von der mitochondrialen Apoptose. Wir haben zwei Mechanismen identifiziert, die für die Induktion der autophagie-abhängigen Nekroptose im Beisein von Dexamethason verantwortlich sein können. Erstens genügten niedrige Konzentrationen von Obatoclax, um die Interaktion zwischen dem Autophagieregulator Beclin-1 mit dem antiapoptotischen Regulator MCL-1 zu stören, welches die Blockade der Autophagie aufheben könnte. Zweitens führte Obatoclax in Kombination mit Dexamethason zu einer starken Inhibition der Aktivität der Kinase mTOR, welche die Induktion der Autophagie reguliert. Mit dieser Kombinationstherapie konnten wir schliesslich in unserem Xenograft-Modell überzeugende präklinische Daten erzeugen, die eine sehr hohe therapeutische Effektivität auf relevante

Patientenproben zeigen. Diese Arbeit bietet eine solide Grundlage, um eine schnelle Umsetzung in die klinische Anwendung zu erreichen. So ist eine klinische Phase-I Studie in Zusammenarbeit mit den wichtigsten europäischen Instituten in Vorbereitung. Unsere Daten schaffen die Basis für die Evaluierung der biologischen Antwort auf die Kombinationsbehandlung mit Obatoclax und Dexamethason in Patienten mit refraktärer ALL und haben entscheidend dazu beigetragen, um dieser klinischen Studie die höchste Priorität in Europa zu gewähren. Wir haben gleichzeitig ein einzigartiges experimentelles System geschaffen, um in einem klinisch hochrelevanten Hintergrund von einem mechanistischen Standpunkt aus die molekularen Ereignisse zu untersuchen, die Autophagie, Nekroptose und Apoptose verbinden. Dieses System kann jetzt ausgebaut werden, um in hochauflösenden Untersuchungen neue Ansätze zur Behandlung von therapie-refraktären Leukämien zu evaluieren und die besten Kandidaten für weitere klinische Studien herauszutriagieren.

Introduction

Chapter 1: Childhood acute lymphoblastic leukemia

Childhood acute lymphoblastic leukemia is the most frequent cancer in childhood. Despite cure rates among children with ALL of 80%, the current therapies have substantial toxic effects and relapse remains a significant problem [7-11]. Leukemia relapse constitutes the fourth most frequent diagnosis in pediatric Oncology in the US and the fifth most frequent diagnosis in the Swiss Childhood Cancer Registry, which underscores the need for new treatment modalities. Leukemia can arise in the B-cell and T-cell lineages and is characterized by abnormal proliferation and a predominance of immature blast cells, blocked in an early differentiation stage (Figure 1) in blood and bone marrow.

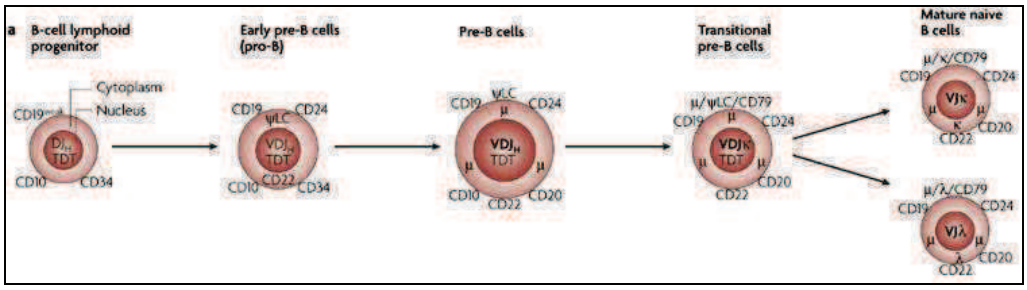

Figure 1: B-cell differentiation stages adapted from [12].

Diagnosis and treatment of childhood ALL depend on the unequivocal detection of a leukemic cell population and identification of the hematopoietic lineage (which includes CD markers and Ig or TCR genes rearrangement) from which the population originates [13-14] (Table 1). Immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements occur during normal lymphoid development and represent a fingerprint for each individual cell and its progeny. Thus, they are widely used as clone-specific markers for clonality analysis of lymphoid cells. They are present in virtually all lymphoid leukemias and may exert specific features as a consequence of the i) age of the patients and/or ii) leukemia subgroup, even though they are not causally linked to the leukemia development. Furthermore, the leukemia

clone-specific Ig/TCR rearrangements may provide information about the time at which the target cell of the leukemia is transformed as well as to the differentiation stage of the precursor cell. Clonal Ig or TCR rearrangements provide essential markers to follow treatment response by Q-PCR to measure minimal residual disease (MRD).

Sub-type	**Leukemic antigen expression (% of positive cases)**								
	CD19	cCD22	CD10	CD7	CD5	cCD3	cIg µ	sIg µ	sIgκ/λ
Early pre-B	100	> 95	95	5	0	0	0	0	0
Pre-B	100	100	>95	0	<2	0	100	100	0
Late pre-B	100	100	50	0	0	0	100	100	0
B-ALL	100	100	50	0	0	0	>95	>95	>95
T-ALL	<5	0	45	100	100	100	0	0	0

Table 1: Immunophenotypes of acute lymphoblastic leukemias adapted from [15].

Acute lymphoblastic leukemia is a complex disease. In the past, discovery of genetic factors contributing to the pathogenesis of ALL were limited by the low resolution of conventional cytogenetic analysis and to molecular analysis of selected individual genes. The analysis of recurrent genetic abnormalities identified a number of important genes that are implicated in the pathogenesis of the disease. Important insights came from twin analysis and detection of lesions that are characteristic for leukemia in neonatal cord blood, even before emergence of the disease [16-19]. This type of analysis strongly suggests the existence of preleukemic clones with primary genetic lesions, which will evolve to full blown disease with additional lesions [20]. A fascinating Darwinian evolutionary model has recently been proposed by Greaves [21]. This model suggests that the first "hit" or first mutation happens prenatally and occurs very commonly, indicating a low penetrance or low evolutionary progression. The acquisition of the critical, secondary CNAs requires some Darwinian selective advantage to expand numbers of cells at risk, like for example TGF beta. The clonal architecture of ALL has been investigated by single cell analysis with multicolor probes to mutant genes. The data do not reveal a linear sequence of mutation acquisition with clonal succession but rather considerable complexity with a tree-like or branching structure of genetically distinct

subclones very reminiscent of Darwin's divergence evolutionary diagram. This pattern has important implications for stem cells in ALL, for the origins of relapse and for therapeutic targeting. With the rapid progress using novel high throughput genomic technologies, a picture of the genetic complexity of ALL is emerging. Genome wide transcriptome analyses have permitted molecular classification of the disease and led to the identification of relevant gene expression signatures characteristic for different subgroups, which can be exploited as discovery tools for hypothesis generation [22-24]. The limitation of gene expression profiling is the difficulty in identifying critical genetic lesions from the long list of deregulated genes characteristic of each cancer or leukemia subtype. In contrast, significant progress is expected from new genetic approaches. Several groups reported studies using high resolution single nucleotide polymorphism (SNP) arrays of diagnostic leukemic samples, which suggest the coexistence of several recurrent genetic aberrations in ALL, mostly small deletions of critical genetic material, but often also chromosomal translocations leading to an oncogenic fusion genes and in some instance seven specific gain of genes. Through the systematic use of SNP arrays we begin to understand which cellular pathways may be targeted by DNA copy number alterations (CNAs). In a majority of cases, master regulators of lymphoid development are deleted, including PAX5, EBF and IKZF1 [25]. Other critical targets appear to include regulators of the cell cycle, tumor suppressor genes (PTEN, BF1, RB1) and regulators of apoptosis (BTG1). Loss of genes regulating B-lymphoid development is present in 60% of pro-B ALL, and this accelerates leukemogenesis in experimental models [23, 25-26], suggesting that these alterations contribute to the process of leukemogenesis in human ALL. Mullighan et al [27] underlined the fact that the average number of CNA in leukemia was generally restricted to a limited number of alterations, about six per cases indicating that in general, ALL is not characterized by gross genomic instability. Furthermore there were significant differences in the number of CNA among ALL. For example, MLL-rearranged

ALL cases had less than one CNA per case, suggesting that few cooperating aberrations are necessary to induce leukemogenesis. In contrast, *BCR-ABL* positive leukemia harbored over six aberrations per case, suggesting the requirement for multiple additional lesions for leukemogenesis. In this last group, one of the most recurrent lesions was found in the IKZF1 gene [25]. Deletion of *IKZF1*, which encodes the transcription factor Ikaros, was detected in 83.7% of *BCR–ABL1* ALL cases, including 76.2% of pediatric and 90.9% of adult *BCR–ABL1* ALL cases (Figure 2). Deletion of *PAX5* occurred in 51% of *BCR–ABL* ALL cases, with the majority also having a deletion of *IKZF1* (95%). This suggests that deletion of *IKZF1* is associated with a very poor outcome in B-cell-progenitor ALL and could contribute to resistance to chemotherapy.

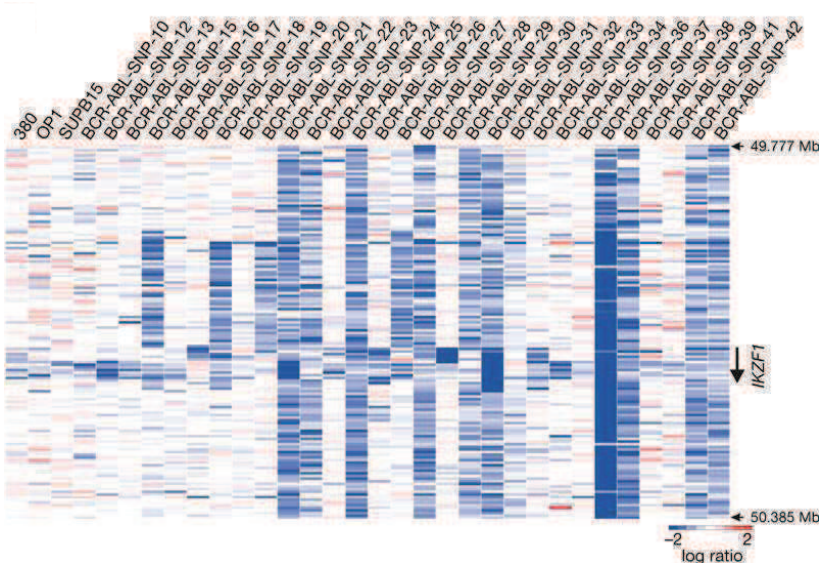

Figure 2: BCR-ABL positive ALL is characterized by the deletion of Ikaros [25]

An integrated approach combining high throughput genomic techniques, deep sequencing of the leukemia genome, and epigenetic profiling (e.g methylation arrays to detect modification in promoter and enhancer regions of candidate genes), will lead to the identification of new lesions in ALL. But linking these data to biological function and clinical significance will require datasets that enable conclusive correlations with clinical outcome data and experimental platforms for functional investigation of the different lesions

Prognostic factors

Several clinical and biologic features have been found to have important prognostic significance in childhood ALL (Table 2), including age, leukocyte count at presentation, immunophenotype, recurrent chromosomal abnormalities, and response to initial therapy [7]. These prognostic factors have been used to stratify patients for optimal therapy adaptation; patients with 'high risk' features have received more intensive treatments, whereas some of the more intensive components of therapy have been modified or eliminated for those children with more favorable presenting features.

Prognostic Factors
Favorable
Hyperdiploidy with trisomies of chromosomes 4, 10, 17
TEL/AML1 fusion-positive
Low end-induction (day 29) MRD (_0.01%)
Low end-induction (day 29) MRD with either double trisonomies (4,10) or TEL/AML1 fusion
Unfavourable
Hypoploidy (less than 45 chromosomes)
Intrachromosomal AML1 amplification
MLL-rearranged
High end-induction (day 29) MRD (>0.01%)
Philadelphia Chromosome positive cells
Ikaros deletion

Table 2: Recently reported prognostically distinctive subsets of children with B-precursor acute lymphoblastic leukemia

Despite the rapid gain of knowledge about recurrent genetic lesions in ALL, so far only a very limited number of these alternations can serve as predictive markers for risk stratification in ALL treatment [28]. For instance, the chromosomal translocation t(9;22) resulting in the BCR-ABL fusion gene, an activated oncogenic kinase, is associated with worse outcome. However, more recent studies in this subgroup demonstrate that assessment of in vivo response to treatment using molecular methods still enables to discriminate among poor and good responders within this subgroup (A. Biondi, AEIOP-BFM study group, personal communication). On the other hand, the translocation t(11;21) leading to the fusion gene ETV6-RUNX1 (TEL-AML1) is clearly associated with favorable prognosis but rare

occurrence of late relapses [29]. It is likely that the new generation of genomic studies will identify additional useful prognostic markers, which would be very valuable to assign patients at risk to experimental therapy early during their first line treatment.

However, so far, assessment of the response to treatment in vivo, by measuring minimal residual disease (MRD) using qPCR after different blocs of chemotherapy remains by far the most powerful approach for patient stratification in ALL [30-31]. To detect MRD junctional regions of Ig or TCR gene rearrangements are suitable PCR targets, because they contain unique sequences owing to the deletion and random insertion of nucleotides during the rearrangement process. Cases with no detectable MRD at 5 weeks and 12 weeks (measured by qPCR) after diagnosis are classified as standard-risk, and those with high levels of MRD (more than 10^{-3}) detectable at both time points, together with patients who have the Ph chromosome, the t(4;11) or initial induction failure, are considered as high-risk of relapse. All the others are considered to have medium-risk leukemia. HR patients can be further subdivided based on persistence of residual disease during consolidation therapy. This group of patients can be considered as very high risk of relapse (VHR) (Figure 3). About half of these patients are steroid resistant.

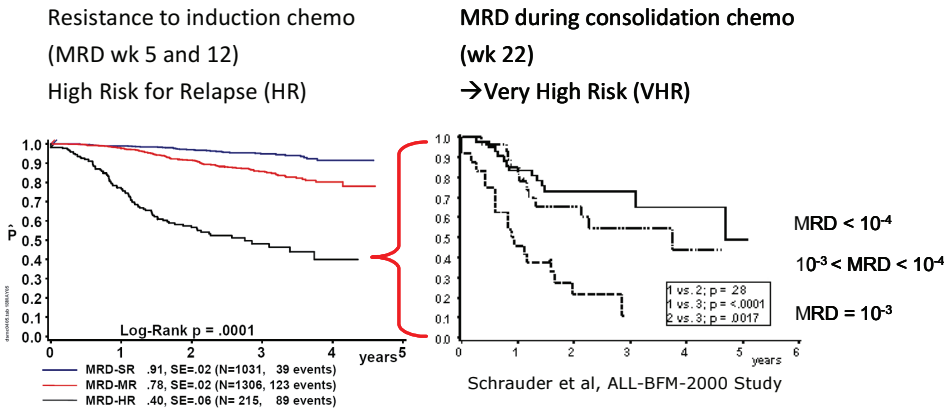

Figure 3: In vivo response to treatment: monitoring of minimal residual disease (MRD)

Of relevance for our work, a poor response to glucocorticoids, as evidenced by persistence of leukemic cells after a window of one week monotherapy with the glucocorticoid (GC) prednisone is a strong independent predictor of poor outcome in childhood ALL [32]. However in most if not all cases, patients with very resistant or refractory disease will have a poor MRD response *in vivo* which correlates with resistance to multiple chemotherapeutic agents *in vitro*, as evidenced by our study (Publication 2) [3]. The importance of glucocorticoids for the control of the disease is also reflected by the fact that a significant proportion of patients with refractory disease are also resistant to glucocorticoids

Molecular mechanism of glucocorticoid action

GCs constitute an essential component of the treatment of lymphoid malignancies, including childhood acute lymphoblastic leukemia [12] due to their ability to induce apoptosis in lymphoid cells [33]. The mechanism of action in ALL is still controversial. The GC-induced apoptotic response is thought to be mediated through the glucocorticoid receptor (GR), a member of the nuclear receptor family of ligand-dependent transcription factors, which is localized in the cytoplasm of unstimulated cells [34]. Ligand binding to the GR results in its dissociation from chaperone proteins that tether the receptor in the cytoplasm, causing a conformational change in the protein that allows nuclear translocation, receptor dimerization, DNA binding, and transactivation [35]. The activated receptor-ligand complex translocates to the nucleus where it binds as a homodimer to specific GC-response elements (GRE) found in the promoter and enhancer regions of GC-responsive genes (Figure 4).

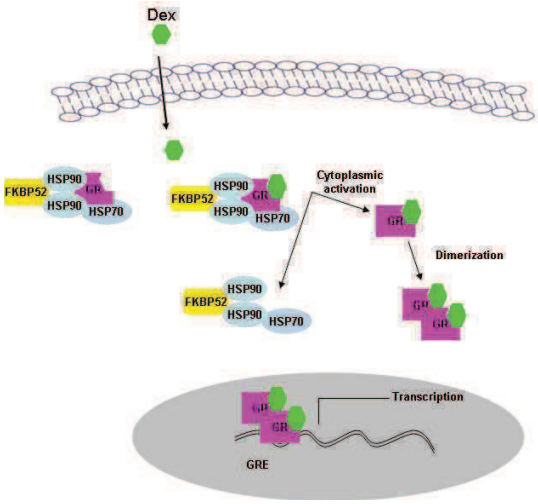

Figure 4: Glucocorticoids: mechanism of action

In lymphoblasts changes in gene expression caused by receptor activation ultimately result in induction of apoptosis [36-37]. GC receptor induced transactivation of GRE containing genes and transrepression of genes via NF-κB and AP-1 interactions are thought to be essential [38-39]. GC induced cell death was shown to occur through the mitochondrial apoptotic pathway and depends on protein synthesis and de novo of target genes [40-41]. Brady *et al*. showed that the effect of GCs is p53 independent [42].

Despite the central role of glucocorticoids in all protocols for the treatment of ALL, the mechanisms by which they act on their target cells, and lesions underlying GC resistance, remain poorly understood [35]. This is due, in part, to lack of suitable experimental systems to study the disease. Due to the limited proliferate capacity of primary leukemia cells *in vitro*, studies investigating GC-resistance have focused to a large extent on laboratory-derived ALL cell lines. Resistance in such cell lines is almost invariably associated with defects in receptor-ligand interactions, such as mutations in the ligand binding domain of the GR [33, 37, 43]. However, similar studies using primary patient samples have detected only isolated cases with somatic mutations in the GR, leading to poor GC-response [44-46]. Although existing data are conflicting, the majority of studies have failed to associate GC-resistance in patient samples with either relative expression of the various isoforms of the receptor [46-47],

polymorphisms within the coding region of the *GR* gene or expression levels of co-chaperone proteins associated with the GR [48].

Molecular mechanism of steroid resistance in childhood ALL

Resistance to glucocorticoids in ALL was proposed to be associated to defect in the apoptotic machinery. Indeed, our work and studies by others [38, 49] have demonstrated that the apoptotic response to glucocorticoids is blocked in steroid resistance. Because several groups reported an increase of mRNA levels of the anti-apoptotic regulator MCL-1 in GC-resistant ALL and based on the observation the upregulation of the pro-apoptotic BCL-2 family protein BIM was altered in GC-resistant ALL, deregulation of the control of the mitochondrial apoptosis by the BCL-2 family could be an important part of the drug resistance mechanism (also see section below and [1]). Furthermore pro-survival signaling by the PI3K-AKT-mTOR axis could contribute to increased resistance to apoptotic signaling in steroid resistant cells. The downstream targets of PI3K-AKT are numerous [50]. In particular, critical interactions between pro-and anti-apoptotic regulators of the BCL-2 family can be modulated by AKT phosphorylation [51-52]. Similarly, AKT regulates the activity of downstream regulators of apoptosis such as the inhibitors of apoptosis protein (IAP) family [53]. Therefore both new agents that interfere with BCL-2-mediated suppression of apoptosis [54] or inhibit signaling events of the PI3K/AKT/mTOR axes [54] may act synergistically with other anti-leukemic agents.

Overactivation of the PI3K/AKT/mTOR pathway

Alterations of the phosphoinositide 3-kinase (PI3K)/AKT pathway (Figure 5), a central integrator of survival signals, were frequently detected in human tumors, including adult lymphoma and ALL [1-2, 55-56]. The mechanisms of AKT activation are multiple. In T-ALL, PTEN phosphatase, negative regulator of PI3K, is often inactivated due to mutations

[57], deletions [58] or epigenetic inactivation. Inactivation of PTEN was shown to play a prominent role in resistance to NOTCH inhibition in T-ALL, an effect that appears to be mediated by AKT [56]. Activation of PI3K-AKT signaling can also occur by mutation of PI3K or AKT in T-ALL [59]. With gene expression profile on primary pre B-ALL, AKT signature was one of the most prominent and was associated with GC-resistance [1]. Furthermore, recent studies underlined the fact that stream regulators of the PI3K/AKT, such as Ras, PTPN11 or FLT3, [60-64] display mutation that might over-activate this pathway. Consistent with this, inactivation of AKT using arsenic trioxide (ATO), re-sensitized steroid resistant cells to dexamethasone. Furthermore, AKT was also shown to regulate members of the Inhibitors of Apoptosis Proteins IAP family, including XIAP and survivin, which are often abnormally expressed in leukemic cells, resulting in a blockade of caspase-dependent cell death pathways [2] [53].

Similarly, in acute myeloid leukemia various mutations were found in this signaling pathway, suggesting that aberrant activation of PI3K/AKT pathways might be of general interest in acute leukemia.

mTOR pathway

mTOR is a central protein involved in cell growth, proliferation, and survival [50]. mTOR can be found in two functionally distinct complexes (see Figure 5). The first, mTORC1 is thought to act downstream of AKT, is known to be inhibited by rapamycin, and regulates mRNA translation [65]. It can also inhibit AKT signaling pathway through a negative feed back loop, which may constitute a mechanism of escape for tumors cells when mTOR inhibitors are used for therapy [66]. The second, mTORC2, is thought to act upstream of AKT phosphorylating it on Ser473, or PKC, regulating cell proliferation and cytoskeleton organization [50] (Figure 5). In a bioinformatics approach, the mTOR inhibitor rapamycin was recently identified as potential modulator of the GC-response [1]. Rapamycin was shown

to improve the response to steroids in GC-resistant cell lines. This effect was proposed to be mediated by a modulation of MCL-1 levels [1]. In the work presented in my thesis, we could demonstrate that the mechanism involved is very different from the model proposed by Armstrong et al. Instead, rapamycin, when given in combination with dexamethasone, triggers the same non-apoptotic cell death pathway than the putative pan BCL2 antagonist obatoclax, which suggests that modulation of mTOR is important for induction of this autophagy-dependent pathway in steroid resistant ALL [67].

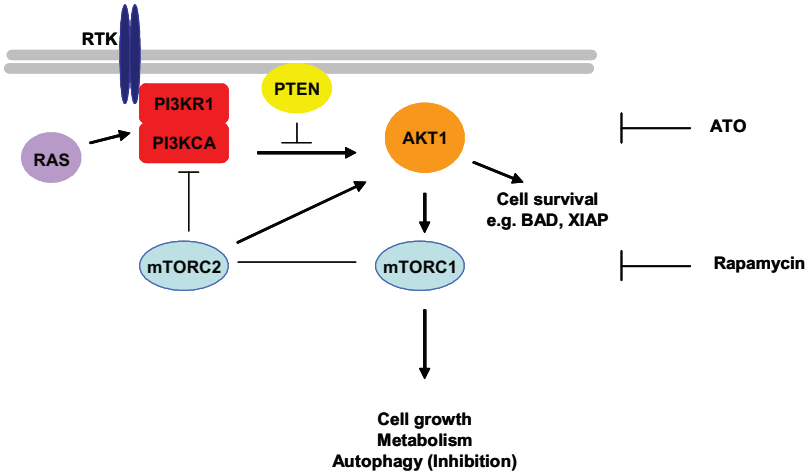

Figure 5: The PI3K/AKT/mTOR pathway. Mutations in this pathway are frequently found in leukemia. Inhibition of one of these components seems to sensitize multiresistant ALL cells to chemotherapeutics adapted from [54].

Chapter 2: Apoptotic cell death

Many chemotherapeutic agents will eventually lead to apoptotic cell death via triggering the apoptotic pathway. Therefore the identification of the factors which contribute to an increased resistance to apoptosis may provide new rationales to develop therapeutic agents for chemomodulation. Apoptosis is a programmed cell death characterized by series of biochemical events that lead to a variety of morphological changes, including membrane blabbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation [68].

This process plays the main role in the regulation of tissue homeostasis, especially in cell systems characterized by a high turn over like the hematopoietic cells [69]. Deregulation of apoptotic process has been described as one of the hallmark of cancer [70]. Mechanisms that confer increased resistance to apoptotic signals may occur at many different levels of apoptotic regulation. Therefore the identification of the factors which contribute to an increased resistance to apoptosis may provide new rationales to develop therapeutic agents for chemomodulation. Apoptosis is distinguished in two different pathways, the extrinsic and the intrinsic pathway.

Intrinsic apoptotic pathway

The intrinsic pathway is under the control of the mitochondria activity [71] and the interplay between pro-apoptotic and anti-apoptotic members of the BCL-2 family at the mitochondria regulate execution of this apoptotic pathway. Depolarization of the mitochondrial membrane induced by apoptotic triggers results in the release of Cytochrome c. Subsequent formation of the apoptosome by recruitment of Apaf1 and Pro-Caspase-9 leads to the activation of

Caspase-9. Activated Caspase-9 in turn activates Caspases-3, 6, and 7, proteases that herald demolition of the cell by cleaving numerous substrate proteins and activating DNases [68].

BCL-2 family of proteins

Key regulators of mitochondria membrane permeabilization are the BCL-2 family proteins. To date, 25 members of the BCL-2 family of proteins have been identified [72]. These proteins are localized to the mitochondria, the smooth endoplasmic reticulum[73], and perinuclear membranes [74]. Overexpression of several anti-apoptotic BCL-2 family proteins has been reported in hematologic malignancies, including leukemia [52]. BCL-2 family proteins are characterized by the presence of up to four relatively short sequence motifs, which are termed BCL-2 homology domains (BH) [72] (Figure 6).

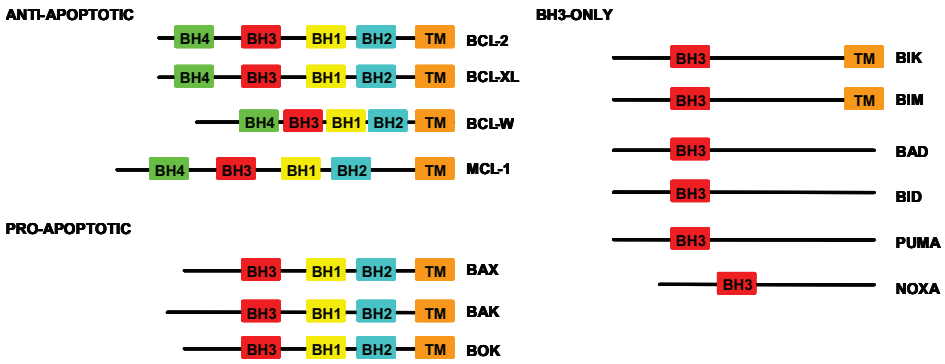

Figure 6: BCL-2 family of proteins

BCL-2 family members can be divided into three subfamilies based on structural and functional features [71]. The anti-apoptotic subfamily contains the BCL-2, BCL-XL, BCL-W, MCL- 1, Bfl1/A-1, and BCL-B proteins, which suppress apoptosis and contain all four BCL-2 homology domains, designated BH1-4. Some pro-apoptotic proteins, such as BAX, BAK, and BOK, contain Bcl-2 homology 1-3 domains and are termed ‘‘multidomain proteins,’’ whereas other pro-apoptotic proteins, such as BIM, BAD, and BID, contain only the BH3 domain and are termed ‘‘BH3-only’’ proteins [75].

The molecular surface of the anti-apoptotic BCL-2 builds a hydrophobic groove, the BH3 binding cleft, which capable of binding the BH3 domain of the pro-apoptotic proteins. The variability of the amino acid composition of both pro-apoptotic BH3 domains as well as the residues lining the BH3-binding groove of the anti-apoptotic proteins dictate highly variable affinities for each specific pair of interactions. These affinities, combined with the spatial and temporal variations of the concentration of each of these protein-protein interactions. This complex interplay will regulate the pro-apoptotic activity of BCL-2 family members [76].

Regulation of the intrinsic apoptotic pathway

The principle site of action of the BCL-2 proteins is at the level of the mitochondria membrane (Figure 7). Anti-apoptotic BCL-2 family members such as BCL-2, -XL or MCL-1 can prevent the activation of the multidomain pro-apoptotic family members, BAX and BAK , either directly, by sequestration other pro-apoptotic BCL-2 proteins, such as BIM, BID or BAD, which could directly activate BAK or BAX [77] (Figure 7). BAK is a mitochondrial membrane protein, while BAX is recruited to the mitochondria from the cytoplasm upon activation of apoptosis. BAX and BAK are required for the permeabilization of the mitochondria membrane [78]. Although the precise mechanism of permeabilization is debated, it results from a conformational change of BAX and BAK which will expose the N-terminal domain followed by the insertion in the mitochondria membrane as homo-oligomerized multimers and the formation of a protein-permeable pore [79].

The BH3-only proteins can be activated by transcriptional induction, post-translational modification or liberation from endogenous inhibitors. They can exert their pro-apoptotic function at least through two different mechanisms. Some BH3-only proteins (so called direct activators such as BID or BIM) may activate the pro-apoptotic proteins directly initiating the apoptotic process either by stimulation of the translocation of BAX on the mitochondria or by a local effect on BAK [80]. Other BH3 (BAD or NOXA) preferentially interact with the anti-

apoptotic proteins dissociating the direct activators from anti-apoptotic proteins which in turn activate BAX and/or BAK by oligomerization and mediate the mitochondria membrane permeabilization [80]. Oligomerization of BAX and BAX induces formation of a pore on the mitochondria membranes through which Cytochrome c and other factors are released [81]. Cytochrome c in the cytoplasm form a complex with Apaf1, resulting in recruitment and activation of Caspase-9. Caspases involved in apoptotic signaling are categorized into initiator and effector caspases [68]. To the family of initiators belong Caspase-9 and 8 which recruit the signal from the Cytochrome c release or the activation of death receptor respectively and activate the effector caspases (Caspase-3, 7, 6) by proteolytic cleavage [82].

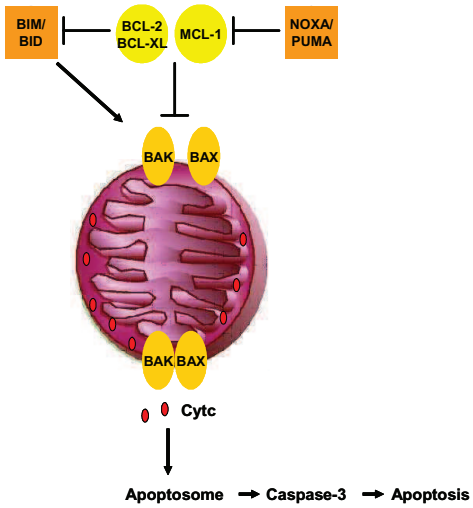

Figure 7: Intrinsic apoptotic signaling pathway

Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is mediated by death receptors [83]. Activation of the death receptor, by ligand binding, can induce apoptosis with or without involvement of mitochondria [83].

The death-receptor pathway is activated when members of the tumor necrosis factor (TNF) super-family bind to death receptors localized at the cell membrane [84]. Ligation of these

receptors initiates the formation of the multiprotein death-inducing signaling complex (DISC) [85].

The DISC complex consists of core proteins (FADD, TRADD, Caspase-8) and components that may mediate crosstalk with other pathways ((Daxx, FAP-1, FLASH, RIP1, FAF-1 and others). DISC formation results in activation of Caspase-8, which can propagate the signal by direct cleavage of the downstream target Caspase-3, or by cleavage of the BH3-only protein BID, which in turn translocates to the mitochondria induces BAX/BAK oligomerization, leading to the formation of the pore from which Cytochrome c is released [85] (Figure 8).

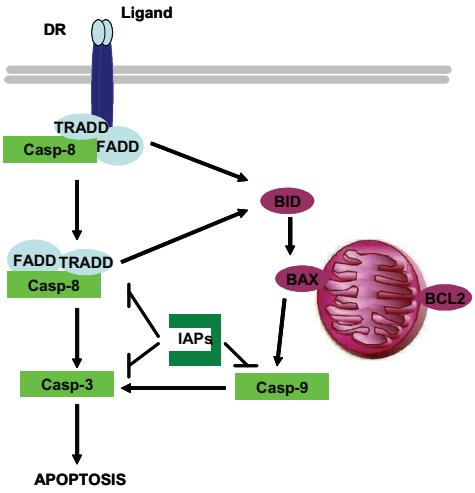

Figure 8: Extrinsic apoptotic signaling pathway.

Activation of death receptor signaling is complex and can induce three different cell responses based on cells types or physiological conditions. Induction of TNFR, for example, can result, as just mentioned, in apoptosis [86], cell survival through NF-kB activation [87], but also in alternative cell deaths [88].

Targeting intrinsic apoptotic pathway

Imbalance between members of the BCL-2 family of proteins has been proposed as an oncogenic mechanism [89-90]. The interaction between the BCL-2 anti-apoptotic with the BH3-only pro-apoptotic proteins is central to the control of apoptosis. Such interactions are

important to maintain the mitochondria membrane polarization. Therefore compounds that would interfere with regulatory interactions of pro- and anti-apoptotic proteins are of therapeutic interest. These interactions are mediated by the conserved BH3 domain and a hydrophobic pocket constituted by the BH1 and BH2 domain of BCL-2 proteins. Proof of principle for such an approach was achieved using a synthetic BID BH3 peptide, stabilized by hydrocarbon stapling (Figure 9) to reconstitute the native helical BH3 interaction domain [91], stapled peptides for the BH3 domain of pro-apoptotic BID displayed cytotoxic activity on ALL cells lines and delayed leukemia progression in a xenograft model [91-92].

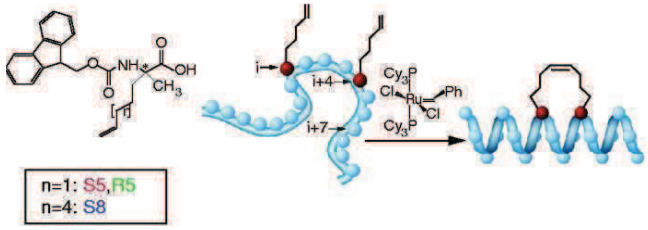

Figure 9: Hydrocarbon-stapled BH3 compounds [92]

In order to restore apoptosis, a number of BCL-2 inhibitors have been developed to target the BH3-mediated interactions [93]. Using nuclear magnetic resonance screening, a small molecule BH3 mimetic, ABT-737, has been identified, that specifically binds into the hydrophobic groove of anti-apoptotic BCL-2 family members [94-95] (Figure 10A). ABT-737 was shown to be effective as single agent in different cancer models including lymphoma and small lung cell carcinoma [96]. Furthermore it was shown to act synergistically with chemotherapeutics and radiation in these cells. A derivate of ABT-737 with better pharmacologic properties, ABT-263, is now in clinical development [97-98]. This agent however does not interfere with all BCL-2 family members. In particular, ABT-737 does not antagonize MCL-1. Several preclinical studies have shown that MCL-1 can mediate resistance to ABT-737 [99-101]. Given the postulated importance of MCL-1 in ALL [99, 102] we suspected that ABT-737 may not be the ideal candidate to be used as chemosensitizer for this disease.

A B

Figure 10: A: Crystal structure of the BCL-XL:ABT-737 complex (right) showing the binding pockets (p2 and p4) for the chloro-biphenyl and thio-phenyl groups of ABT-737 [95]. B: Predicted orientation of obatoclax within the BH3 binding groove of BCL-2 [103].

We therefore focused our attention on another compound, which was identified from a screen for small molecules that can interact with MCL-1 [103]. This small molecule, obatoclax [104], is an indole pyrrole derivative with very hydrophobic properties, which so far have prevented the exact characterization of the interaction with the supposed drug targets *in vitro*. In particular, there is no crystal structure available of this compound in complex with a BCL-2 family member [103]. The predicted interaction with BCL-XL is depicted in Figure 10B. Biochemical and functional data using cell lines strongly suggest that obatoclax can disrupt the complexes between pro- and anti-apoptotic BCL-2 family members, in particular between MCL-1 and BAX [101, 103, 105-108]. As reported in publication 2 [3], obatoclax displays strong anti-leukemic activity at a sub-micromolar concentration. We showed that in combination with conventional chemotherapeutic agents, such as doxorubicine, cytarabine, or vincristine, sub-cytotoxic concentrations of these agents was sufficient to restore mitochondrial apoptosis. But unexpectedly, we discovered that this compound was also capable of very specific induction of a non-apoptotic cell death pathway in combination with glucocorticoids. This indicates that obatoclax interferes with critical cellular targets at the intersection of pro-survival and pro-death pathways.

Targeting the extrinsic apoptotic pathway

The extrinsic pathway begins outside the cell through the activation of death receptors on the cell surface. These are triggered by specific molecules known as pro-apoptotic ligands. Pre-clinical models showed clearly that the death receptor ligand TRAIL is effective in various cancer types including leukemia [109-114]. Resistance to TRAIL or to the CD95 ligand in leukemia is mainly due to inhibition of Caspases through members of the Inhibitor of Apoptosis IAP family in particular XIAP [115]. Fulda et al. showed that combination of a small molecule IAP inhibitor with ligands of the death receptor, leukemia cells were sensitized to CD95 or TRAIL [116-118]. TRAIL is already in clinical trial for non small cell lung cancer as single agent or in combination. This approach could therefore be of interest for combination therapy in ALL.

Chapter 3: Alternative cell death pathways in a context of defective apoptosis

Besides apoptosis, two other alternative cell death pathways have been described, namely autophagy-dependent cell death, and programmed necrosis (necroptosis) [119-120]. These non-apoptotic forms of cell death have been described in cellular models in the context of defective apoptosis [120]. Apoptosis is ultimately executed by caspases. The use of caspase inhibitors has revealed the existence of alternative backup cell death programs for apoptosis in cell lines. The broad-spectrum caspase inhibitor zVAD-fmk modulates the three major types of cell death. Addition of zVAD-fmk blocks apoptotic cell death, sensitizes cells to necrotic cell death, and induces autophagic cell death.

Autophagy

In healthy cells, autophagy is a highly regulated cellular process by which cells recycle their own non essential, redundant, or damaged organelles and macromolecular components [121-122]. It is under the control of ATG genes, which are conserved throughout evolution, suggesting their importance. During this process part of the cytoplasm is sequestered into autophagosomes and finally delivered to lysosomes for bulk degradation [123]. Beyond this homeostatic function [124-127], autophagy is a process by which the cells adapt their metabolism to starvation [128-131]. By catabolizing proteins and macromolecules, autophagy generates metabolites that are used by the cells as substrate for the bioenergetics needs. The functional relationship between autophagy and apoptosis is complex. Depending on the cellular context, autophagy occurs as a stress response to pro-apoptotoc signals and thereby can interfere with the action of chemotherapeutic agents or radiotherapy. The mechanisms by which autophagy promotes cell survival are not restricted to its role in maintaining cellular energy homeostasis during starvation. Autophagy is also involved in removing damaged

mitochondria and other organelles. In this context autophagy can promote cell survival also during aging, infection disease and neurodegenerative process. In fact increasing evidence underlines the cytoprotective role of autophagy that allows to increase tumor cell survival under conditions of metabolic stress and hypoxia as well as to escape chemotherapy-induced cell death. In breast cancer for example induction of autophagy protects cells from trastuzumab treatment [132]. However there is also clear evidence for the role of autophagy in programmed cell death [133-134] (see below).

Regulation of the autophagic pathway

The execution of autophagy is mediated by evolutionarily conserved proteins known as the autophagy-related (ATG) proteins [135]. So far, the systematic analysis of the expression patterns and transcriptional regulation of ATG genes has remained incompletely defined. Among the approximately 30 autophagy-related (ATG) genes identified so far, there are two ubiquitin-like proteins, ATG12 and ATG8. Analogous to ubiquitination, ATG12 is conjugated to ATG5 by ATG7—an E1-like protein—and ATG10—an E2-like protein. Similarly, ATG7 and ATG3 are the respective E1-like and E2-like proteins that mediate the conjugation of Atg8 to phosphatidylethanolamine. Both ATG12–ATG5 and ATG8 localize to the developing autophagosome. The ATG12–ATG5 conjugate facilitates the lipidation of ATG8 and directs its correct subcellular localization. ATG8–phosphatidylethanolamine is probably a scaffold protein that supports membrane expansion and the amount present correlates with the size of autophagosomes [135-136](Figure 11).

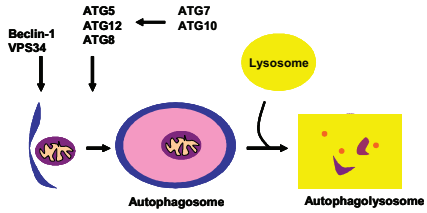

Figure 11: Localization of the ATG proteins on the autophagosome

Regulation of autophagy induction through mTOR

mTOR is an upstream negative regulator of this process [137-138]. Availability of cellular amino acids, especially branched chain amino acids such as leucine, or glucose levels regulate mTOR activity. In fact nutrient starvation, or low glucose availability inhibits the kinase activity of mTOR and induces autophagy. From this observation many studies have been performed in order to understand the connection between mTOR and autophagy. It has been shown that mTOR phosphorylates an upstream autophagy protein ATG13 inhibiting the formation of a complex in with UKL1 [139-140]. Inhibition of mTORC1 by rapamycin or by starvation, leads to dephosphorylation of ULK, and Atg13 in human cells which induces autophagy. These studies suggested that ULK and Atg13 could act as direct targets of mTORC1 [139-140].

An important element in the nutrient-signaling pathway upstream of mTORC1 involves hVps34 also named PI3KIII (phosphoinositide 3-kinase class III), a lipid kinase conserved throughout eukaryotes and key regulator of autophagy [141-142]. Deficiency of the mammalian hVps34 suppressed leucine-responsive activation of mTORC1, suggesting that hVps34 may act upstream of mTORC1 signaling [143] (Figure 12). Consistent with an important role of mTOR in the control of autophagy, a small molecule screen, performed to identify new pharmacologic inducers of autophagy identified mainly mTOR inhibitors [144].

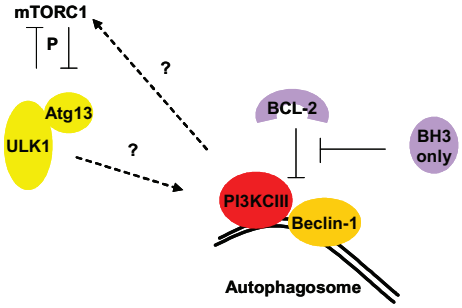

Figure 12: Regulation of autophagy by mTOR and during vesicles nucleation

The autophagy gene Beclin-1 is a critical regulator.

Beclin-1 was found in protein complexes that mediate very early steps of the autophagic process. Deletion of Beclin-1 prevents autophagy, underscoring the importance of this autophagy regulator [145]. Interestingly, evidence from mouse models suggests that Beclin-1 can act as a haploinsufficient tumor suppressor, since Beclin-$1^{+/-}$ mutant mice suffer from a high incidence of spontaneous tumors [146]. This suggests that Beclin-1 could be involved in pathways that control tumor progression, in particular by contributing to cell death. Interestingly, Beclin-1 was also found to be a BH3-only protein and as consequence possibly to be regulated by BCL-2 anti-apoptotic proteins. The interaction of Beclin-1 with BCL-2 anti-apoptotic proteins results in sequestration of Beclin-1 from PI3K class III complex and inhibition of autophagy [133]. The interaction of Beclin-1 and BCL-2 is thought to be weaker than the interaction of BCL-2 with other BH3 proteins [147-148]. Functionally, there is some evidence that disruption of Beclin-1 from BCL-2 by BAD or by micromolar concentrations of the BH3-mimetic ABT-737 can lead to induction of autophagy [149], suggesting the possibility that the BCL-2 family control mechanisms at the intersection of apoptosis and autophagy control.

In the work submitted in this thesis, we have shown that sub-cytotoxic concentrations of obatoclax were sufficient to disrupt a complex between endogenous Beclin-1 and MCL-1 and that this phenomenon was associated with steroid re-sensitization. Our observation supports a model in which MCL-1 is part of a protein complex that is critical for the control of autophagy.

Evidence for autophagy-dependent cell death

In several reports functional data were provided that support a role for autophagy in cell death. In the central nervous system, ATG7 deficiency protected neurons from caspase-dependent and caspase-independent cell death after hypoxic/ischemic brain injury [150]. In

human glioblastoma, knockdown of the autophagy genes ATG1 or ATG5 prevented the cytotoxic effect of cannabinoids, which induce autophagy-dependent cell death in via an mTORC1 dependent pathway [151]. In *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, and in BCL-2 over-expressing MEFs, cell death triggered by etoposide or staurosporine was dependent on autophagy genes Beclin-1 and ATG-5 [145]. Consistent with this, inhibition of cysteine proteases can cause autophagic cell death in selected cell types. For example in U937 monocytoid cells and macrophages that were treated with lipopolysaccharide, the inhibition of cysteine proteases with z-VAD-fmk induced autophagic cell death, which was attenuated by the RNA interference (RNAi)-mediated knockdown of beclin-1 [152]. This effect is likely to involve the combined inhibition of one or several caspases, including Caspase-8, as well as that of a protease from another class of cysteine proteases, most probably a calpain [153]. Furthermore, inhibition of caspases induces autophagy also in Jurkat cell lines treated with tumor necrosis factor alpha (TNFa). Furthermore recent insight from drosophila genetics indicate that the control of autophagy via mTOR is critical to induce regression of the salivary glands, which provides the first evidence for a critical role for autophagy-dependent cell death in development [154]. We now provide the first clear evidence for a role for autophagic cell death in cancer.

A programmed form of necrotic cell death is relevant for tissue homeostasis and cancer

A form of programmed necrosis has been reported by several groups to occur in circumstances where the normal apoptotic response is defective [155-158]. This alternative cell death pathway appears to be regulated by the receptor interacting protein (RIP1) [4]. RIP1 is a central kinase associated with death receptor-induced signaling complexes and represent a molecular switch between cell death and survival [155]. Tschopp et al discovered that in active lymphoblasts treated with FAS, the caspase inhibitor zVAD induced a form of

cell death with necrotic features, regulated by the kinase activity of RIP1. Consistent with this, studies in RIP1-deficient Jurkat cells, demonstrated that necroptosis, induced by death receptors ligands TNF, FAS or TRAIL in apoptotic-deficient conditions, depends on the presence of RIP1 [155, 159-161].

RIP1 is essential for pro-survival and pro-death signaling

RIP1 was first identified as a critical mediator of pro-survival signals. Consistent with the role of RIP1 in cell survival, mice lacking RIP1 display extensive apoptosis [162]. This effect appears to be related to the death receptor pathway. Deletion of RIP1 increases sensitivity to TNF induced cell death. RIP1 mediates this pro-survival effect via activation of NF-kB [163]. Activation of the TNF receptor, results in the recruitment of a protein complex to the receptor that includes RIP1 [163-164], which in turn results in activation of a wide range of target genes, which also include anti-apoptotic regulators (XIAP, cIAP and BCL-XL) [165-167], Cellular inhibitors of apoptosis (cIAPs) have been shown to mediateRIP1 ubiquitination in this complex [165-166] (Figure 13).

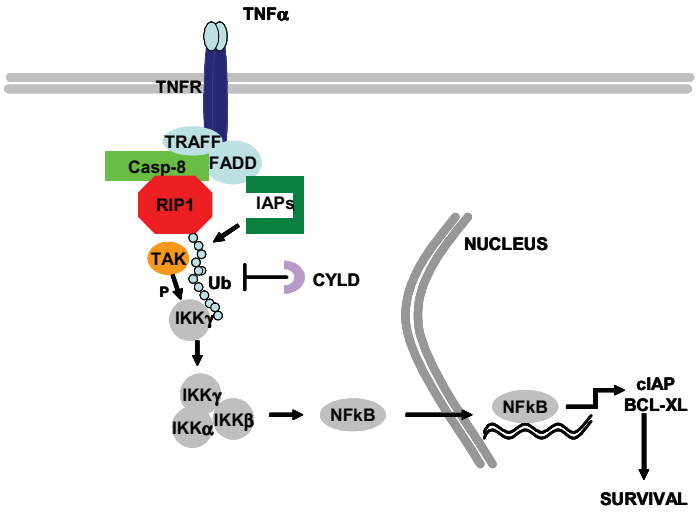

Figure 13: Regulation of NF-kB by TNFR

Several groups have shown that RIP1 kinase function mediates the activation of a non-apoptotic cell death pathway in cells that are deficient for effector caspase function [155, 157,

159]. A very specific inhibitor of RIP1 kinase has been identified (NEC-1), which constitutes a very useful tool for the investigation of RIP1 kinase function in vivo [157]. Besides RIP1, a functional RNA interference screen identified a number of genes that are required for programmed necrosis, some of which overlap with genes that also control apoptosis [168]. As expected this screen also identified the RIP1 regulator CYLD, whose essential function for the necroptotic pathway was validated by *si*RNA knock down experiments. Collectively, available data suggest that the ubiquitination status of RIP1 is a critical component of the molecular switch between pro-survival and pro-death signaling via RIP1. The molecular mechanisms of this switch are still poorly understood. The different RIP1 functions may involve different protein complexes. Activation of the death receptor induces internalization and formation of secondary cytosolic complexes, one dependent on TRADD adaptor protein (complex IIA) and one dependent on RIP1(Complex IIB) (Figure 14). The molecular events that result in activation of a necroptotic pathway when caspase-dependent cell death is blocked however remain to be elucidated. So far, it appears that a complex formed by FADD, Caspase-8, RIP1 and RIP3 induces necroptosis [169-170]. In fact it has been shown that interaction between RIP1 and RIP3 is important in order to induce necroptosis. RIP3 has been reported to act positively with many metabolic enzymes such as PYGL, GLUD1, and GLUL, this interaction induces formation of reactive oxidative stress (ROS) due to increased oxidative phosphorylation. Increased level of ROS induces methabolic stress which leads then to necroptosis [170].

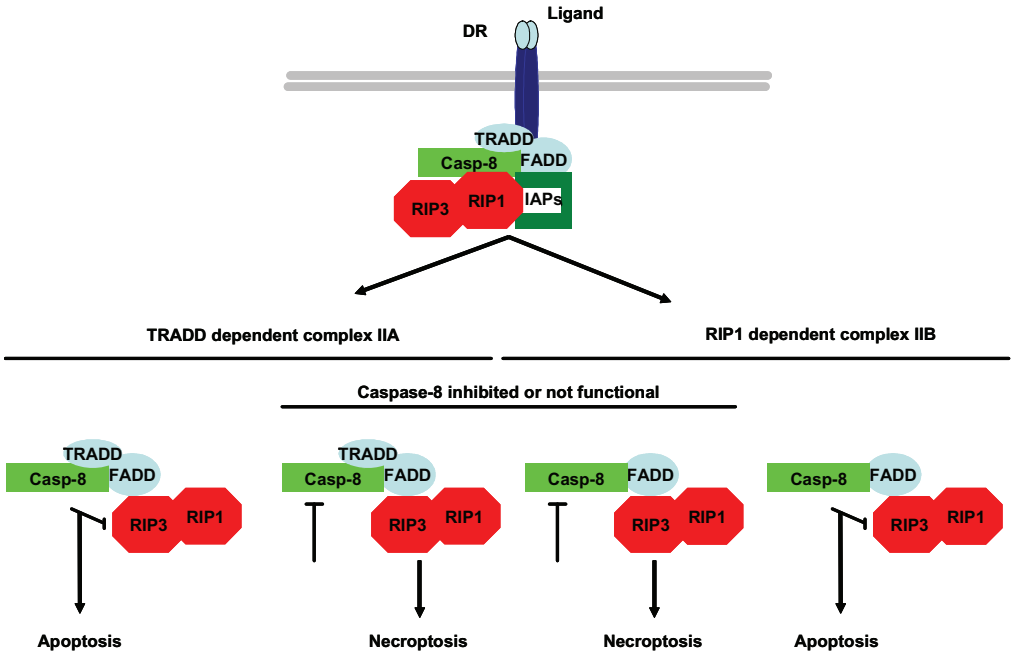

Figure 14: Death receptor induces autophagy or necroptosis

Here I present our unanticipated discovery, that re-sensitization to the major anti-leukemic drug dexamethasone in highly resistant ALL is absolutely dependent on early induction of the autophagy pathway and strictly required RIP1 kinase dependent activation of necroptosis. We provide functional data for the importance of both pathway by showing complete rescue from re-sensitization after knock down of genes that are essential for apoptosis (ATG7, Beclin-1) and for necroptosis (RIP1, CYLD). We also show that modulation of mTOR is critical for this cell death pathway, which is consistent with our earlier data, demonstrating a pivotal role for the hyperactivity of the AKT pathway in drug resistant ALL. This work places cellular events that modulate autophagy and the necroptotic pathway in a new perspective in the context of highly resistant disease and point toward major perturbations in drug resistant ALL at the intersection of metabolic pathways, apoptosis and necroptosis. We will discuss the implications of our findings from the mechanistic and molecular standpoint, and the immediate implications for the clinical development of this promising therapeutic strategy.

Subject of Investigation

Childhood acute lymphoblastic leukemia is the most recurrent cancer in childhood. Nowadays, with the current treatments, about 80% of the patients are cured. However 20% of the patients are refractory to initial chemotherapy or they relapsed. Resistance to the first seven days of treatment with steroids is one of the most important prognostic factors of poor outcome. Steroid resistance has been suggested to be due to an apoptotic blockade, resulting from either overactivation of PI3KI or overexpression of BCL-2 anti-apoptotic proteins. We aimed to investigate new strategies in order to overcome steroid resistance in ALL.

1) Low-dose arsenic trioxide sensitizes glucocorticoid-resistant acute lymphoblastic leukemia cells to dexamethasone via an Akt-dependent pathway. (Article 1)

In this (second authorship) paper we characterized and revealed the mechanism of how inhibition of the PI3K/AKT pathway by using arsenic trioxide (ATO) sensitizes GC-resistant ALL cells to dexamethasone. Combination of ATO with dexamethasone induced inhibition of AKT, which affected its downstream targets such as the BH3-only protein BAD and the endogenous Caspase inhibitor XIAP.

2) Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. (Article 2)

In this (First authorship) paper, I evaluated the potential of a pan-BCL2 inhibitor called obatoclax, to sensitize steroid resistant cells to dexamethasone. The combination treatment resulted in activation of autophagy-dependent necroptosis. I also showed that obatoclax was able to sensitize primary cells of VHR-ALL patients to daunorubicine, vincristine and cytarabin. In this context cell death had apoptotic features. I also showed that the combination treatment with dexamethasone and obatoclax was effective *in vivo* in our refractory leukemia xenograft model.

This article is the main part of my work and thus will be the essential part in the discussion and outlook section.

Contribution to the papers:

Publication 1:

For this publication my contribute was to prove that the decrease in cell prolipheration, observed in MTT assay, in steroid resistant cell lines upon treatment with dexamethasone and arsenic trioxide (ATO), was due to increase of cell death. I performed annexin V PI stainig and caspase 3 activation assay.

Publication 2:

For this publication on which is based my PhD project, my contribution was:

Experimentally:
I performed all the experiments published except for:
Figure 6 A/B/C
The experiments showed in this figure were performed by Dr.Beat Bornhauser.
Figure 8 C: Animals were transplanted intrafemorarly by Dr. Beat Bornhauser.
Animal treatment was shared between me and Dr. Beat Bornhauser.

Planning:
Experiments were planned and designed by me, Dr. Bornhauser and PD. Bourquin
The papers were written by Dr. Bornhauser and PD. Bourquin.

Results

Low-dose arsenic trioxide sensitizes glucocorticoid-resistant acute lymphoblastic leukemia cells to dexamethasone via an AKT-dependent pathway.

Bornhauser BC, Bonapace L, Lindholm D, Martinez R, Cario G, Schrappe M, Niggli FK, Schäfer BW, Bourquin JP.

Blood. 2007 Sep 15;110(6):2084-91.

Abstract

Incorporation of apoptosis-inducing agents into current therapeutic regimens is an attractive strategy to improve treatment for drug-resistant leukemia. We tested the potential of arsenic trioxide (ATO) to restore the response to dexamethasone in glucocorticoid (GC)-resistant acute lymphoblastic leukemia (ALL). Low-dose ATO markedly increased *in vitro* GC sensitivity of ALL cells from T-cell and precursor B-cell ALL patients with poor in vivo response to prednisone. In GC-resistant cell lines, this effect was mediated, at least in part, by inhibition of AKT and affecting downstream AKT targets such as BAD, a pro-apoptotic BCL-2 family member, and the X-linked inhibitor of apoptosis protein (XIAP). Combination of ATO and dexamethasone resulted in increased BAD and rapid down-regulation of XIAP, while levels of the anti-apoptotic regulator MCL-1 remained unchanged. Expression of dominant-active AKT, reduction of BAD expression by RNA interference, or overexpression of XIAP abrogated the sensitizing effect of ATO. The inhibitory effect of XIAP overexpression was reduced when the AKT phosphorylation site was mutated (XIAP-S87A). These data suggest that the combination of ATO and glucocorticoids could be advantageous in GC-resistant ALL and reveal additional targets for the evaluation of new anti-leukemic agents.

For detailed information see attached manuscript 1.

Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance.

Bonapace L*, Bornhauser BC*, Schmitz M, Cario G, Ziegler U, Niggli FK, Schäfer BW, Schrappe M, Stanulla M, Bourquin JP.

J Clin Invest. 2010 Apr;120(4):1310-23. *equal contribution

Abstract

In vivo resistance to first-line chemotherapy, including to glucocorticoids, is a strong predictor of poor outcome in children with acute lymphoblastic leukemia (ALL). Modulation of cell death regulators represents an attractive strategy for subverting such drug resistance. Here we report complete re-sensitization of multidrug-resistant childhood ALL cells to glucocorticoids and other cytotoxic agents with subcytotoxic concentrations of obatoclax, a putative antagonist of BCL-2 family members. The reversal of GC-resistance occurred through rapid activation of autophagy-dependent necroptosis, which bypassed the block in mitochondrial apoptosis. This effect was associated with dissociation of the autophagy inducer beclin-1 from the anti-apoptotic BCL-2 family member myeloid cell leukemia sequence 1 (MCL-1) and with a marked decrease in mammalian target of rapamycin (mTOR) activity. Consistent with a protective role for mTOR in GC-resistance in childhood ALL, combination of rapamycin with the glucocorticoid dexamethasone triggered autophagy-dependent cell death, with characteristic features of necroptosis. Execution of cell death, but not induction of autophagy, was strictly dependent on expression of receptor-interacting protein (RIP1) kinase and cylindromatosis (turban tumor syndrome) (CYLD), two key regulators of necroptosis. Accordingly, both inhibition of RIP-1 and interference with CYLD restored GC-resistance completely. Together with evidence for a chemosensitizing activity of obatoclax in vivo, our data provide a compelling rationale for clinical translation of this pharmacological approach into treatments for patients with refractory ALL.

For detailed information see attached manuscript 2.

Discussion & Outlook

In my thesis I describe two pharmacological approaches to specifically bypass the apoptotic blockade to chemotherapy in multidrug resistant ALL cells. From a mechanistic standpoint, our results indicate that in the context of resistant cancer cells, interference with mechanisms that are at the intersection of pro-survival and pro-death regulation can restore programmed cell death to anti-leukemic drugs.

The molecular mechanisms of drug resistance are still poorly understood in ALL. Resistance to apoptotic stimuli constitutes one of the hallmarks in cancer [171-172]. We and others [3, 38] provide evidence for a blockade in the apoptotic response to dexamethasone in steroid resistant ALL cells. Despite decades of research, the anti-leukemic mechanism of glucocorticoids remains largely unknown. Glucocorticoids induce the intrinsic apoptosis pathway in ALL cells [173-174]. Resistance to Glucocorticoids could occur at many levels. Glucocorticoid resistance in primary ALL cells does not seem to be related to decreased expression or mutations of the GR [175]. Consistent with this, a screen for nucleotide variations using a PCR/single-strand conformational polymorphism sequencing strategy demonstrated that polymorphisms but not somatic mutations in the GR gene coding region occur in leukemic blasts of children with ALL. These data suggest that these genetic variations are not a major contributor for differences in cellular response to glucocorticoids in childhood ALL [45].

The principal gateway to mitochondrial apoptosis is controlled by the BCL-2 family of proteins. Anti-apoptotic activity of BCL-2 family members can be modulated by increasing transcription or stability [176-181]. Accordingly, imbalance between pro- and anti-apoptotic BCL2 proteins has been shown to contribute to increased resistance to anti-leukemic therapy [182-183]. For instance, the anti-apoptotic protein BCL2 was required for maintenance of

leukemia in a mouse model [90]. In the specific context of steroid resistance in childhood ALL, anti-apoptotic MCL-1 was shown to be overexpressed in primary ALL cells as part of the gene expression signature associated with *in vitro* GC resistance [184]. Deletion of BIM and/or PUMA or overexpression of BCL-2 or MCL-1 conferred GC-resistance in murine lymphoid cells [89]. Furthermore, dexamethasone was suggested to cause cell death through an increase of BIM levels in steroid-sensitive ALL cell lines [185]. To evaluate the involvement of the BCL-2 anti-apoptotic proteins in steroid resistant ALL cells with the idea to target these, many compounds were proposed. Peptides and BH3 mimetic compounds are becoming increasingly popular in a pipeline of products as pro-apoptotic anti-cancer drugs. The stabilized alpha helix stapled peptides (SAHB, [91]) represent an intriguing way to gain entry to the complex cascades of protein-protein interactions in the cell-death pathway. This is because they can theoretically retain structural and functional properties of native apoptotic proteins.

The potential of these peptides to induce apoptosis in steroid resistant ALL cells was demonstrated *in vitro* and *in vivo* [92]. Native BH3-only proteins exhibit selectivity in binding pro-survival members, as do stapled peptides and small molecules that block these interactions. In particular it was proposed that BAD binds BCL-2 but not MCL-1. Based on this knowledge, our unpublished data demonstrate that only BID and BIM but not BAD SAHB can induce cell death in steroid resistant ALL as single agent but also in combination with dexamethasone. Moreover, low dose of obatoclax, but not ABT-737, sensitizes steroid resistant cells to dexamethasone. These data underline the possible role of MCL-1 in a context of GC-resistance in ALL and suggest that obatoclax sensitizes through a mechanism which may involve inhibition of MCL-1. Surprisingly, we found that steroid re-sensitization by obatoclax did not occur through activation of the classical intrinsic apoptosis pathway, since no mitochondria membrane depolarization occurred, Cytochrome c was not released

and Caspases 3 and 9 were not activated. Instead, cell death occurred through autophagy-dependent induction of programmed necrosis, also referred to as necroptosis. It is well known that BCL-2 family of protein can regulate autophagy. Nutrient starvation, which is a potent physiologic inducer of autophagy, can stimulate the dissociation of Beclin- 1 from its inhibitors, by activating BH3-only proteins (such as BAD) [149]. Maiuri et al demonstrated that ABT-737, as well as BAD, is able to disrupt the interaction between BCL-2 and Beclin-1, resulting in autophagy induction [149]. Consistent with this, we demonstrated that in steroid resistant ALL cell lines MCL-1 binds Beclin-1. Furthermore we found that subcytotoxic concentration of obatoclax, but not ABT-737 can disrupt this complex, suggesting that obatoclax could sensitize steroid resistant ALL cells by inhibiting MCL-1 and inducing autophagy. More experiments are needed in order to understand whether the effect of obatoclax on the protein containing MCL-1 and Beclin-1 results from a direct interaction or is secondary to other mechanisms and whether or not true BH3 mimesis is involved in this process. In our hands, functional experiments with MCL-1 knock down and overpression were not conclusive due to strong effects on cell cycle and apoptosis. Taken together these results suggest that obatoclax sensitizes steroid resistant cells to dexamethasone through a mechanism which involves autophagy and requires MCL-1.

Autophagy is known as physiological mechanism which can rescue cells from apoptosis. More controversial is its involvement in cell death. Autophagy can occur to rescue cells from cell death. Indeed it was reported in many papers that both pharmacological inhibitors of autophagy and siRNAs that target essential modulators of the autophagic machinery have been shown to sensitize cancer cells to a wide spectrum of stress conditions, including (but not limited to): glucose and amino acid deprivation [186-187] growth factor withdrawal [188]; detachment from the extracellular matrix (i.e., anoikis) [189]; estrogen receptor

antagonism with tamoxifen [190]; radiation therapy [191]. Autophagy can also be a mechanism of cell death in a context of apoptosis-deficiency. Consistent with this, as mentioned in the introduction, in *Bax/Bak* DKO MEF, representing a background of apoptosis blockade, etoposide induces autophagic cell death. The fact that oncoproteins and tumor suppressor proteins from the BCL-2 family also control autophagy further substantiates the notion that tumor development may be favored by autophagy inhibition. We discovered that in steroid resistant ALL combination treatment with dexamethasone and obatoclax induces autophagy-dependent cell death. In this case induction of autophagy seems to be required to induce cell death. Consistent with this, pharmacological inhibition of autophagy, by using the PI3KCIII inhibitor 3-MA or Bafilomycin, or genetically inhibition, by downregulation of Beclin-1 and ATG7, completely blocked steroid sensitization in GC-resistant cell lines and refractory ALL primary cells. Moreover, combination of dexamethasone and obatoclax inhibited clonogenic growth of GC-resistant ALL cells.

Interestingly, we observed that in steroid resistant ALL cells, sensitization to dexamethasone, by different drugs occurred through induction of autophagy. Rapamycin [1] and arsenic trioxide [2] have been shown to be GC-sensitizers in steroid resistant ALL. It is known that rapamycin induces autophagy through inhibition of mTOR [1, 192-193], whereas ATO was shown to induce autophagic cell death in apoptosis-resistant glioma cell lines [194]. We also observed (data not published) that the autophagy inhibitor 3-MA prevents the steroid-sensitizing effect of these two drugs. It is important to note that rapamycin and ATO both interfere with the PI3K/AKT/mTOR axis. Overactivation of the AKT pathway was proposed to contribute to chemoresistance in ALL. Constitutive activation of this pathway induces mTOR activity and therefore inhibition of autophagy in resistant disease. We therefore hypothesized that GC-resistant cells could be primed for mTOR-controlled autophagy. Indeed we reported that treatment with dexamethasone and obatoclax induces

dephosphorylation of a downstream target of mTOR, s6 protein, in resistant cells. This effect appears to be specific to the combination with glucocorticoids and induction of autophagy-dependent cell death. Indeed, induction of apoptotic cell death by the combination of obatoclax with other cytotoxic agents was not associated with modulation of mTOR activity in refractory ALL. Taken together these data suggest that in steroid resistant ALL cells, induction of autophagy-dependent cell death is peculiar for the combination treatment with dexamethasone and obatoclax. Interestingly during Drosophila morphogenesis, the steroid ecdysone induces autophagic cell death in salivary glands, suggesting as well a role for steroids in inducing an autophagy-dependent cell death pathway. However other investigations have to be performed in order to clarify this point and the mechanism by which obatoclax induces apoptosis in combination with other cytotoxic agents. But the assessment of the phosphorylation status of mTOR targets may constitute a valuable biomarker to determine the biological response in patients treated with the combination of obatoclax and dexamethasone.

We have shown that induction of autophagy is required for execution of programmed necrosis in ALL cells. This provides one of the first evidence for the importance of this emerging pathway in cancer. This non-apoptotic form of cell death appears to be tightly controlled [168]. Necroptosis constitutes a form of programmed cell death, because it does depend on the function of specific regulators [157]. So far this phenomenon has only been observed in a cellular context where apoptosis is blocked. It has long been known that lymphocytes induce non-apoptotic cell death upon alteration of specific apoptotic proteins such as Caspase-8 or FADD. In lymphoid cells that fail to activate Caspase-8 during T cell mitogenesis, a hyperactive autophagic state was induced followed by programmed necrotic cell death [120] which was dependent on RIP-1 kinase [155, 158, 195]. Consistent with this,

Degterev and his group showed that silencing of RIP1 or inhibiting its kinase activity with Necrostatin-1 (NEC-1) [157, 196] blocks necroptosis induced by TNF and zVAD [157, 197] in lymphoid cells. Here we show by electron microscopy that cell death induced by dexamethasone and obatoclax is characterized by autophagosomes formation and necrotic features. This pathway did strictly depend on RIP1, because knock down or inhibition of RIP1 reverted steroid sensitization completely. Ubiquitination status of RIP1 appears to determine the switch from pro-death to pro-survival signaling. One of the main regulator of this process is the deubiquitinase enzyme CYLD [198]. Consistent with previous information showing that RIP1 needs to be ubiquitinated to interact with survival targets [199], we confirmed that by silencing CYLD, necroptosis induced by double treatment was prevented. This is consistent with functional data by others, detecting RIP1 and CYLD as essential genes for the necroptotic pathway [168].

Our studies highlight that necroptosis and autophagy are interconnected and that stimulation of autophagy is necessary to induce necroptotic cell death. It is still unclear which genes act upstream of RIP-1 and which are the targets of RIP-1 kinase activity. Interestingly, the cellular sensitivity to necroptosis appears to be connected to cellular pathways that mediate innate immunity.

As ALL cells derive from early progenitors of lymphoid cells, it is conceivable that activation of pathways that have evolved as a response to pathogens could also be essential for oncogenesis. Necroptosis has been proposed as a salvage cell death pathway in cells that would be prevented to undergo apoptosis in the event of a viral infection [200].Consistent with the connection of necroptosis and innate immunity, cell death induced by activation of TLR3 in L292 cells in presence of INF requires RIP1 kinase activity to induce necroptosis. Bell et al proposed that as with other forms of danger (in this case damage organelles,

nutrient starvation, macromolecular structures) as detected by TLRs, autophagosome formation itself lead to the activation of signaling pathway that allows the cells to respond to intracellular stressors, if the source of "danger" overrides autophagy, cells respond in induction of cell death [169].Cell death can have apoptotic features or necroptotic in case of block in the apoptotic pathway. Consistent with this, Hitomi et al showed that in the context of caspase inhibition, stimulation of the TNF receptor pathway triggered necroptosis in L929 mouse fibrosarcoma and in Jurkat ALL cells. In this model, knocking down or inhibiting RIP1 kinase activity, autophagy is prevented, which suggest a crosstalk between the two pathways. There is only very limited knowledge about a molecular connection between autophagy and necroptosis. Recent studies demonstrated the presence of a complex including elements of the DISC complex (FADD and Caspase-8) together with RIP1 on preparation of autophagosome membranes, in the association with ATG5/13 [201-202]. We propose that such a complex could possibly be central in mediating apoptotic cell death. In absence of the activation of Caspase-8 by cleavage in ALL cells undergoing necroptosis, this raises the question of a non-apoptotic function of Caspase-8 in a complex that includes RIP1.

We plan to further investigate the molecular mechanisms involved in necroptosis induction in the context of steroid resistant ALL by two different approaches. First we will take advantage of RIP-1 and Caspase 8 deficient ALL cells to perform which we can rescue with engineered RIP-1 or Caspase-8 genes. This will enable us to perform tandem affinity purification of multiprotein complexes for mass spectrometry to detect specific changes of complexes that contain RIP-1 and Caspase-8 on and off obatoclax and dexamethasone in steroid resistant cells. Second we plan to optimize transfection of primary ALL cells to perform synthetic lethal screens in order to identify the genes that are essential for induction of the necroptotic pathway with this treatment. In our leukemia xenograft mouse model of refractory ALL

primary cells, we demonstrated that the treatment with obatoclax in combination with dexamethasone was effective. We observed durable remission with one year of follow-up, indicative of strong antileukemic activity.

The results of my thesis show that steroid in combination with obatoclax induces autophagy-dependent necroptosis. It is likely that obatoclax act as steroid sensitizer through induction of autophagy, furthermore, in this context mTOR activity decreases. Obatoclax sensitizes also to other chemotherapeutics, but the resulting cell death has apoptotic features suggesting that the mitochondria or the death receptor are involved. It is still unclear how RIP1 kinase is primed to induce necroptosis in this context. In Figure 15 the summary of my research is represented.

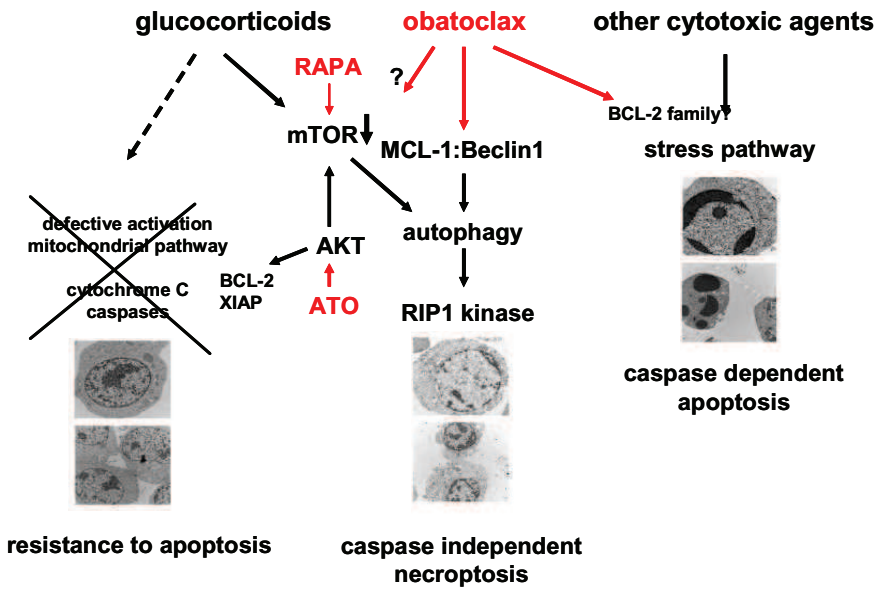

Figure 15: Summary of the research

From a clinical standpoint, our data provide compelling evidence for the evaluation of chemomodulation using obatoclax in refractory ALL. This work has raised sustained interest within the community of experts in the field of pediatric leukemia. With the support of the drug resistant disease committee of the international BFM study group, a clinical protocol for a phase I trial with obatoclax has been developed by my mentor, Dr. Jean-Pierre Bourquin. The goal of this trial is to establish the safety of the combination of obatoclax with dexamethasone in heavily pretreated patients with relapsed or refractory ALL. The proposal

has obtained first priority at the European level and will be conducted in collaboration with the European ITCC consortium (Innovative Therapies for Childhood Cancer) and under co-sponsorship of the University of Zurich and of the Cancer Research UK Clinical Trials Unit, University of Birmingham, which coordinates all clinical trials in pediatric oncology in the UK. This will be a multicenter European trial, including 7-10 experienced larger oncology centers with experience in phase I trials.

Based on pharmacokinetic data from adult phase I trials with obatoclax, we know that obatoclax concentrations that were sufficient for chemosensitization of resistant ALL cells in co-culture on bone marrow stromal cells can be achieved at doses that are inferior to the maximal tolerated dose in adults with hematological malignancies [203-204] which constitutes the basis to establish dose levels for the pediatric trial. An overview of the study design is presented in Figure 16. Besides ruling out limiting toxicities, it will be essential to provide evidence for biological activity of the combination of obatoclax and dexamethasone *in vivo* in patients. Indeed it will be impossible to recruit enough patients to obtain conclusive data about the clinical efficacy of this treatment. It is critical to obtain first safety data and pharmacokinetic data in children to then design a phase II treatment element to assess the effectivity of this combination in patients with relapsed ALL. The current concept shared among experts in the field would be to perform a therapeutic window to test this element in relapsed ALL patients before they get started with the current best available relapse treatment protocol. *In vivo* evidence for chemosensitizing activity will strongly support the design of a phase II study. Furthermore, a phase I trial is currently initiated by the North American by the Childrens Oncology Group (COG) to test the safety of obatoclax in combination with doxorubicine and vincristine. The two pediatric trials are designed to be complementary. Valuable information will be available for the combination of obatoclax with dexamethasone

and with other non steroidal drugs. Together this will enable the design of an experimental bloc with 3 to 4 drugs for the treatment of high risk relapsed ALL. Furthermore the European study will test a new dose schedule for obatoclax with the intention to provide more constant exposure to the drug over one week, which is the typical length for chemotherapy elements in clinical protocols for the treatment of relapsed ALL [205].

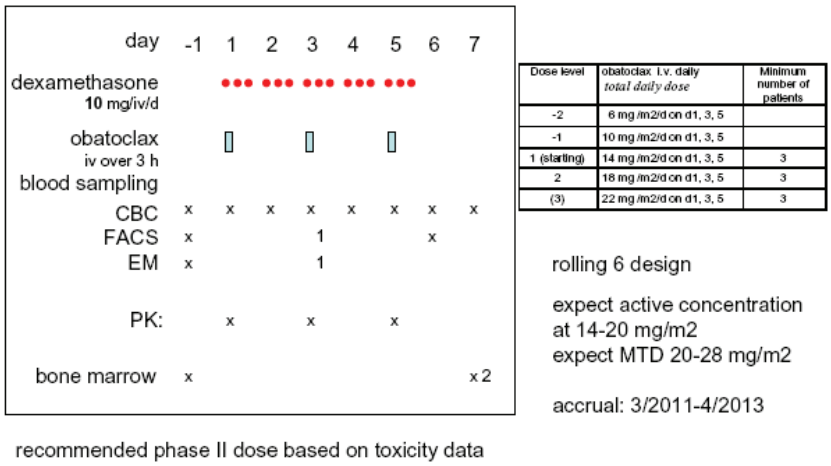

Figure 16: Design of the clinical trial. The proposed design allows for rapid dose escalation and to perform essential pharmacokinetic and biological studies.

One critical part of the clinical phase I study as planned here concerns the assessment of biological activity of obatoclax *in vitro* and *in vivo*. Apart from following the effect of the treatment with obatoclax on leukemia cells by flow cytometry in treated patients (see Figure 16), it will be essential to demonstrate *in vivo* activity with respect to induction of cell death in leukemia cells. We therefore aim to provide the basis for the evaluation of a biological response in treated patients and for the development of an in vitro assay to monitor response to treatment.

Combination of obatoclax and dexamethasone results in induction of autophagy-dependent necroptosis. Morphological features allow identifying this cell death mode using transmission electron microscopy. We are currently developing this method to monitor ALL cells in the peripheral blood from patients. The idea is to fix samples from bone marrow punctures of

from peripheral blood, before isolation of lymphocytes by FACS based on forward-side scatter properties.

The electron microscopy protocol which I have developed during my PhD study will serve as basis for protocol optimization (publication 2, Figure 7). In a multicentric clinical setting, samples from peripheral blood and bone marrow will have to be fixed in the hematology laboratory of the treating center and shipped to our laboratory for microscopy. The critical aspect of protocol optimization will be to determine experimental conditions to concentrate the leucocytes from the patient's blood for imaging. I have established a cell line from a patient de novo resistant ALL patient after passage in mice which we can maintain and treat in liquid suspension cultures. With this model I will compare different approaches to prepare whole blood with different amounts of ALL cells for TEM. This approach will enable us to determine the minimal amount of leucocytes that are required for reliable detection of the necroptotic features by TEM. Indeed, the mean blast count after the first week of prednisone monotherapy in first line therapy is 0.36 $x10^6$/ml peripheral blood (Martin Zimmerman, statistician of the BFM study group). In the phase I trial the average blast count is expected to be higher than in patients at initial presentation and the time point to study the biological response to obatoclax treatment will be earlier. It is therefore realistic to test if 0.5-1.0 $x10^5$ cells/ml are sufficient for TEM. Because the method will be used for a proof of principle study on a limited number of patients, the methodology needs to be sensitive, but we can afford time consuming and extensive microscopy. In a second step the setting of the clinical trial can be simulated using our leukemia xenograft model. Based on our current data from experiments with ALL cell lines, we estimate that the best time point to detect necroptotic features in patients may be after 72 hours of treatment. Xenografted mice will be treated as outlined in Figure 16 once more than 10% of leukemia cells are detectable in the peripheral blood by FACS (Figure 17).

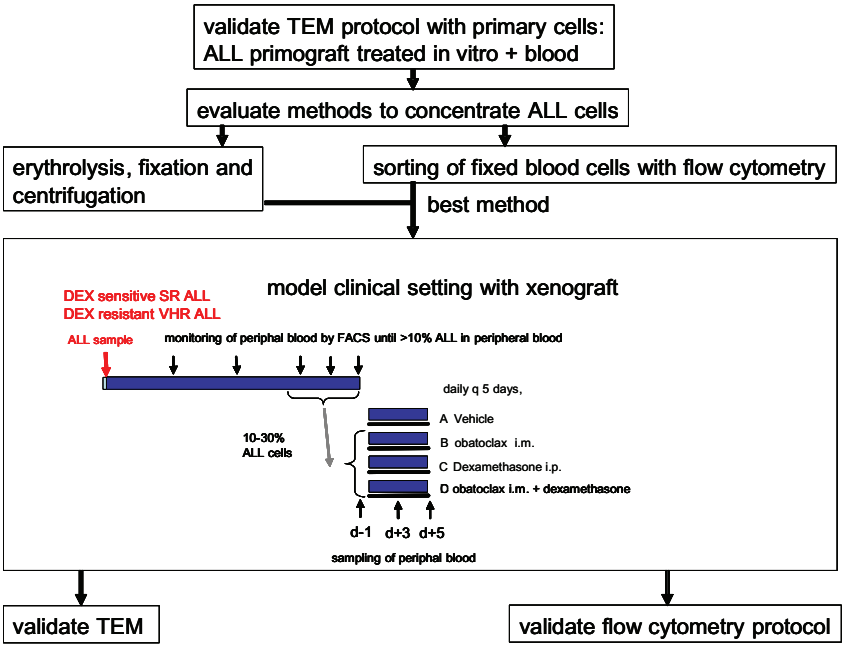

Figure 17: Workflow to model *in vivo* monitoring of treatment response

The second implication from my work for this clinical trial derives from my observation that only the combination of obatoclax and dexamethasone specifically results in strong inhibition of mTOR activity in resistant ALL patients. This opens a possibility to detect a specific modulation of a cellular marker using intracellular flow cytometry, which is a well established method by now [206]. This approach has been use to identify predictive phospho-flow patterns that correlated with outcome in adult AML [207]. Inactivation of mTOR signaling could be used to assess treatment response *in vitro*, on ALL cells from patients that would be eligible for this type of experimental therapy. We have a large series of relevant samples in our xenograft bank to perform pilot experiments and establish a correlation between phosphor-flow patterns after perturbation with drugs *in vitro* and the *in vitro* response to combination treatment. Provided a reproducible pattern emerges, phospho-flow cytometry could also be used to monitor the effect of the treatment on ALL cells during the trial.

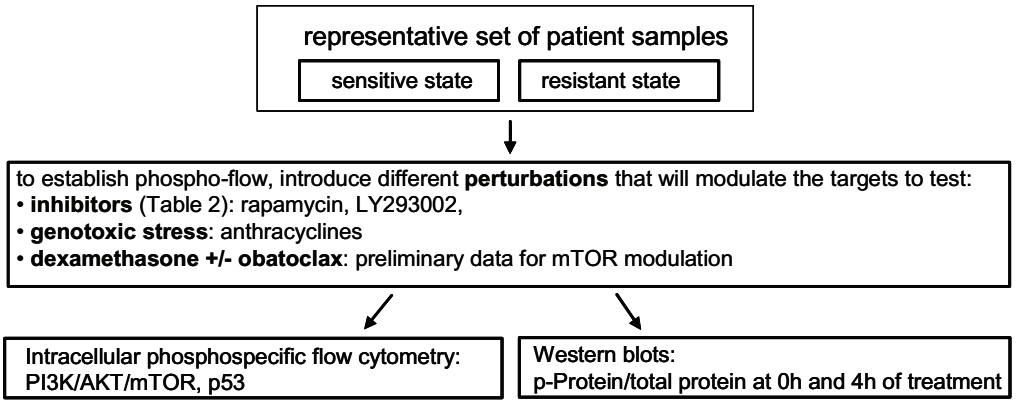

Figure 18: Work flow to establish the phospho-flow cytometry panels.

Collectively, the work performed during my doctoral studies contributed significantly to the development of a novel therapeutic strategy for drug resistant ALL. We have built a leukemia xenograft model that enable to perform functional studies with the leukemia samples that are directly relevant for translational research. We discovered a new cell death pathway that is specifically triggered in the context of steroid resistant leukemia. This observation opens an entirely new avenue for the investigation of steroid resistance in ALL. We provide convincing evidence for effective chemosensitization of different chemotherapeutic agents in refractory ALL. This effect was also validated with *in vivo* experiments using ALL cells from highly resistant patients. Finally, this work constituted the basis for the development of a phase I clinical trial, the first step for the clinical development of this approach.

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Curriculum Vitae

Name:	LAURA BONAPACE
Date of birth:	April 7th, 1979
Place of birth:	Tione di Trento, Italy
Nationality:	Italian
Education:	

since 09/2006	**University of Zurich**, Participant in the Cancer Network PhD program
since 04/2006	**University of Zurich / Kinderspital Zurich** (PhD thesis)
10/2005-04/2006	**Universita' degli studi di Bologna** Stage in department of "fisiologia umana" Reduced brain cell proliferation in Ts65Dn mice, An animal model of Down syndrome
10/2005	**Universita' degli studi di Bologna** finished study of biology with summa cum laude
10/2004 – 10/2005	**Università degli studi di Bologna** Diploma thesis in the group of Prof. R.Bartesaghi (Fisiologia Umana) " Reduced brain cell proliferation in Ts65Dn mice, An animal model of Down syndrome."
07/1998	**High School Liceo-ScientificoL.Da Vinci** of Tione di Trento study of biology ("Diplom")

Publication list

1. **Laura Bonapace**[1], Beat C. Bornhauser[1], Maike Schmitz, Gunnar Cario, Urs Ziegle, Felix Niggli, Beat W. Schäfer, Martin Schrappe, Martin Stanulla, and Jean-Pierre Bourquin
   Induction of autophagy-dependent necroptosis is required to overcome glucocorticoid resistance in acute lymphoblastic leukemia
   JCI 2010 April; 120 (4) 1310-1323 .
   [1] Equal contribution

2. Bornhauser BC, **Bonapace L**, Lindholm D, Martinez R, Cario G, Schrappe M, Niggli FK, Schäfer BW, Bourquin JPB Low-dose arsenic trioxide sensitizes glucocorticoid-

resistant acute lymphoblastic leukemia cells to dexamethasone via an Akt-dependent pathway. Blood. 2007 Sep 15;110(6):2084-91

3. Contestabile A, Fila T, **Bonapace L**, Ceccarelli C, Bonasoni P, Santini D, Bartesaghi R, Ciani
   Cell cycle alteration and decreased cell proliferation in the hippocampal dentate gyrus and in the neocortical germinal matrix of features with Down syndrome and in Ts65Dn mice. Hippocampus. 2007; 17(8):665-78

Selected Poster Presentations, Talks and Awards

1. **Kind-Philipp-Preises für pädiatrisch-onkologische Forschung** 2009

2. **Young Investigator Award**: Charles Rodolphe Brupbacher February 2009 Zurich. "Induction of autophagy-deapandent cell death is required to restore steroid sensitivity in steroid-resistant ALL

3. **European Cell Death Organization** ECDO Bern (September 2008) The BH3 mimetic obatoclax overcomes glucocorticoid-resistance by activating autophagic cell death

4. International Symposium ACUTE LEUKEMIAS XII: Biology and treatment strategies (February 2008) " The small molecule Obatoclax restores the response to Dexamethasone in Glucocorticoid-resistant ALL through induction of Autophagy

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Manuscripts

Contribution to the papers:

Publication 1:

For this publication my contribute was to prove that the decrease in cell prolipheration, observed in MTT assay, in steroid resistant cell lines upon treatment with dexamethasone and arsenic trioxide (ATO), was due to increase of cell death. So I performed annexin V PI stainig and caspase 3 activation assay.

Publication 2:

For this publication on which is based my PhD project, my contribution was:

Experimentally:
I performed all the experiments published except for:
Figure 6 A/B/C
The experiments showed in this figure were performed by Beat Bornhauser.
Figure 8 C: Animals were transplanted intrafemorarly by Dr. Beat Bornhauser.
Animal treatment was shared between me and Dr. Beat Bornhauser.

Planning:

Experiment were planned and designed by me, Dr. Bornhauser and PD. Bourquin

Low-dose arsenic trioxide sensitizes glucocorticoid-resistant acute lymphoblastic leukemia cells to dexamethasone via an Akt-dependent pathway

Beat C. Bornhauser,[1] Laura Bonapace,[1] Dan Lindholm,[2] Rodrigo Martinez,[2] Gunnar Cario,[3] Martin Schrappe,[3] Felix K. Niggli,[1] Beat W. Schäfer,[1] and Jean-Pierre Bourquin[1]

[1]Department of Oncology, Children's Hospital, University of Zurich, Zurich, Switzerland; [2]Department of Neuroscience, Biomedical Center, University of Uppsala, Uppsala, Sweden; [3]Department of Pediatrics, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

Incorporation of apoptosis-inducing agents into current therapeutic regimens is an attractive strategy to improve treatment for drug-resistant leukemia. We tested the potential of arsenic trioxide (ATO) to restore the response to dexamethasone in glucocorticoid (GC)–resistant acute lymphoblastic leukemia (ALL). Low-dose ATO markedly increased in vitro GC sensitivity of ALL cells from T-cell and precursor B-cell ALL patients with poor in vivo response to prednisone. In GC-resistant cell lines, this effect was mediated, at least in part, by inhibition of Akt and affecting downstream Akt targets such as Bad, a proapoptotic Bcl-2 family member, and the X-linked inhibitor of apoptosis protein (XIAP). Combination of ATO and dexamethasone resulted in increased Bad and rapid down-regulation of XIAP, while levels of the antiapoptotic regulator Mcl-1 remained unchanged. Expression of dominant-active Akt, reduction of Bad expression by RNA interference, or overexpression of XIAP abrogated the sensitizing effect of ATO. The inhibitory effect of XIAP overexpression was reduced when the Akt phosphorylation site was mutated (XIAP-S87A). These data suggest that the combination of ATO and glucocorticoids could be advantageous in GC-resistant ALL and reveal additional targets for the evaluation of new antileukemic agents. (Blood. 2007; 110:2084-2091)

Introduction

Poor response to 7-day monotherapy with prednisone is one of the strongest predictors of adverse outcome for the treatment of childhood acute lymphoblastic leukemia (ALL).[1] Despite intensive research efforts, the mechanisms underlying glucocorticoid (GC) resistance in ALL are still poorly understood. GC resistance is unlikely to be GC-receptor (GR)–mediated because mutations of the GR are uncommon and alterations in GR expression or function have not been detected in primary leukemia samples from GC-resistant patients.[2-4]

Because most agents used in ALL treatment, including glucocorticoids, induce programmed cell death (apoptosis), mechanisms that lead to an increased antiapoptotic state in leukemic cells may constitute an alternative explanation for GC resistance in ALL. There is increasing evidence for abnormal apoptotic signaling in GC-resistant ALL, involving members of the Bcl-2 family of regulators of the intrinsic apoptotic pathway.[5] The antiapoptotic Bcl-2 family member Mcl-1 was overexpressed in primary ALL cells as part of the gene expression signature associated with in vitro GC resistance.[6,7] Expression levels of the proapoptotic Bcl-2 family protein Bim were increased after exposure to GC in sensitive cells.[8-11] Interestingly, induction of Bim was markedly attenuated in response to prednisone in GC-resistant primary ALL cells that were amplified by xenotransplantation.[2] Deletion of Bim or Puma conferred GC resistance of normal lymphoid tissues in mice.[12] These studies suggest that an imbalance between proapoptotic and antiapoptotic regulators may be involved in the resistance mechanisms. In agreement with this hypothesis, overexpression of Bcl-2 or Mcl-1 rendered murine lymphoid cells resistant to dexamethasone.[13]

An imbalance that favors an antiapoptotic state in leukemic cells may result from constitutive activation of certain prosurvival pathways. Alterations of the phosphoinositide 3-kinase (PI3K)/Akt pathway, a central integrator of survival signals, were frequently detected in human tumors,[14] including adult lymphoma and ALL cell lines.[15-17] Akt is able to modulate a number of apoptotic regulators directly. Phosphorylation of the Bcl-2 family member Bad by Akt was shown to induce Bad sequestration by protein 14-3-3, thereby blocking its proapoptotic function.[18] At the postmitochondrial level, Akt phosphorylation stabilized levels of XIAP, a member of the inhibitor of apoptosis protein (IAP) family that inhibits caspases-3, -7, and -9.[19] Response of lymphoma cells to the PI3K/Akt inhibitor LY294002 correlated with a decrease in XIAP protein levels,[17] suggesting that XIAP may be a relevant target in lymphoma. An important downstream effector of Akt is mTOR (mammalian target of rapamycin).[20] Inhibition of mTOR with rapamycin sensitized multiple myeloma and ALL cells to dexamethasone,[21,22] underscoring the importance of this pathway. Interestingly, the effect of rapamycin in ALL cell lines was associated with a marked decrease of the antiapoptotic regulator Mcl-1.[21]

Among therapeutic agents with a potential to inhibit Akt, arsenic trioxide (ATO) is of interest for several reasons. The safety profile of ATO for the effective treatment of acute promyelocytic leukemia (APL) is well established.[23-25] ATO was shown to bear relevant cytotoxic activity on T-ALL cell lines,[26,27] which may

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involve a broad range of mechanisms.[28] Depending on the type of malignancy studied, induction of oxidative stress and reactive oxygen species production by ATO was associated with a perturbation of the NF-κB[29,30] or the PI3K/Akt[15] pathways. In ALL cell lines, down-regulation of Akt by RNA interference increased the cytotoxic effect of ATO, indicating that inhibition of this pathway by ATO may be relevant for its effect on lymphoid malignancies.[15] Given the broad range of mechanisms by which ATO could interfere with apoptosis resistance, we decided to evaluate this agent in combination with dexamethasone in GC-resistant ALL. Here we provide evidence that low-dose ATO treatment acts as a GC sensitizer in ALL and that this effect is dependent on inhibition of Akt. These data are consistent with a role for XIAP and Bad, 2 direct targets of Akt, in ATO-induced GC sensitization.

Patients, materials, and methods

Cell culture

CEM-C7-14 and CEM-C1-15 were kindly provided by Dr E. B. Thompson (University of Texas Medical Branch, Galveston, TX). We will refer to these lines as CEM-C7 and CEM-C1 in the text. The CEM, MOLT-4 (no. CRL-1582; ATCC, Manassas, VA), and Jurkat (no. TIB-152; ATCC) cell lines were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin (both from Invitrogen, Carlsbad, CA).

Reagents

ATO was obtained from Sigma (Buchs, Switzerland); PD98059, from VWR international (Dietikon, Switzerland); and dexamethasone, from Mepha Pharma (Aesch, Switzerland). Rabbit antibodies were from Cell Signaling Technology (Danvers, MA): anti-PARP (1:1000), anti-Bim (1:1000), anti-Bid (1:1000), anti-XIAP (1:1000), anti-Akt (1:1000), anti-pAkt (pSer473, 1:1000), anti-Bad (1:1000), rabbit anti-pBad (pSer136, 1:1000), anti–Bcl-X_L (1:1000), anti-PUMA (1:1000), and anti-cIAP1 (1:1000). Rabbit anti-cIAP2 (1:700) and antisurvivin were from R&D systems (Minneapolis, MN). Rabbit antiactin (1:3000) and rabbit anti–Mcl-1 (1:3000) were from Sigma, and mouse anti–Bcl-2 was from Dako (Glostrup, Denmark).

Expression vectors, siRNA, and transfection

Full-length human XIAP cDNA was subcloned into the expression vector pEGFP-C1. Mutagenesis was performed by polymerase chain reaction (PCR) on a subcloned XIAP fragment. For XIAP-S87A, we used the primers 5′-CACAGGAAAGTAGCCCCAAATTGCAG-3′ and 5′-AATTTGGGGCTACTTTCCTGTGTCTTCC-3′; for the XIAP-S87E construct, the primers 5′-CACAGGAAAGTAGAGCCAAATTGCAG-3′ and 5′-AATTTGGCTCTACTTTCCTGTGTCTTCC-3′ were used. The pEGFP-XIAP constructs and a myristoylated/palmitylated-HA-Akt construct[31] were transfected using nucleofection (Amaxa, Cologne, Germany). Transfection efficiency was controlled using the pEGFP plasmid (Clontech, Mountain View, CA) where appropriate. For nucleofection, 30 nM Bad siRNA (Bad nucleotides 817-837 and 1033-1051, NM_004322; Silencer siRNA library, cat no. 81 820; Ambion, Austin, TX) was used (solution v and Amaxa program T-016).

Patient samples

Primary ALL cells were recovered in small portions from banked frozen presentation samples on dry ice using a sterile scalpel and allowed to recover in RPMI supplemented with 10% FCS, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (Sigma) for 4 hours. Cell viability as determined by trypan blue exclusion after thawing was between 50% and 85%. Cells (5×10^4) were incubated in 100 μL medium in round-bottom 96-well plates. Patients were enrolled in the ongoing ALL-BFM 2000 protocol and had given informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the institutional review board of the Medical School Hannover for these studies. This IRB is the central IRB for all participating centers in trial ALL-BFM 2000. In addition, the studies have been approved by the local IRBs of each participating institution, including the University Children's Hospital Zurich. According to ALL-BFM criteria, prednisone good-response (PGR) was defined as the reduction of leukemic blasts in the peripheral blood to lower than 1000 per microliter on day 8 after 7 days of monotherapy with prednisone. Minimal residual disease (MRD) was measured after induction therapy on treatment day 33 and after induction at week 12. MRD high risk is defined as a persistent high MRD load at week 12 (10^{-3} or greater).

Treatment, MTT assay, and determination of apoptosis

Experiments were performed in 96-well plates with 2×10^4 cells per well. Viability of cells was assessed using the cell proliferation kit (MTT) (Roche Applied Sciences, Rotkreuz, Switzerland). The minimal absorbance of control wells was OD 0.8. For primary patient samples, the trypan blue dye exclusion assay was used to determine the effect of treatment on cell viability. Annexin-V stainings were performed with FITC-labeled annexin-V and 1 mg/mL propidium iodide (BD Biosciences, San Jose, CA). Briefly, treated 10^5 cells were washed in cold PBS and then resuspended in 100 μL binding buffer (10 mM HEPES/NaOH, pH 7.4, and 140 mM $NaCl_2$). Annexin-V–FITC and PI (5 μL each) were added and incubated at 4° in the dark for 30 minutes before flow cytometry analysis (Beckman Coulter Cytomics FC500; Hialeah, FL). Caspase activity was detected by CaspGLOW Red Active Caspase-3 Staining Kit (Alexis Platform, Lausen, Switzerland) according to the manufacturer's instructions. Briefly, 1 μL specific substrate for caspase-3 (Red-DEVD-FMK) was added to 10^5 treated cells in 300 μL of medium for 1 hour at 37°C and enzymatic cleavage was analyzed by flow cytometry.

All experiments were performed in triplicate and repeated at least 3 times. Data analysis was done with the GraphPad prism software (San Diego, CA) and statistical analysis using the Student *t* test.

Determination of cellular reactive oxygen species (ROS)

The generation of ROS was analyzed using 2′-dichlorofluorescein diacetate (DCFDA; Sigma). Cells (2×10^4) were incubated with ATO for 5 hours. After incubation with 20 μM DCFDA for 4 hours at 37°C, signals were analyzed by fluorometry using filters for excitation at 485/20 nm and emission at 530/35 nm. The results were normalized to total protein expression levels and are shown as arbitrary fluorescent units.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

For Western blotting, whole-cell extracts were prepared from 1×10^6 cells using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton-X-100, 0.1% SDS) supplemented with complete mini protease inhibitor cocktail (Roche Applied Sciences) and 1 mM sodium orthovanadate (Sigma) for 20 minutes on ice. For detection of primary antibodies, we used horseradish peroxidase–labeled goat antirabbit or antimouse antibodies from Pierce Biotechnology (Rockford, IL). Signal detection was performed using the chemiluminescence substrate from Pierce Biotechnology, directly scanned with the ChemiGenius imaging system (SynGene, Cambridge, United Kingdom) and quantified with the GeneTools 3.1 image analysis software (SynGene).

Results

ATO augments the response to dexamethasone in glucocorticoid-resistant acute lymphoblastic cell lines

In order to evaluate the potential of ATO for the treatment of glucocorticoid (GC)–resistant ALL, we selected a panel of T-ALL cell lines that show different sensitivity to GC and established dose-response curves for ATO and dexamethasone (Table 1). As expected from previous reports,[15,27,32] the GC-sensitive CEM-C7, and the GC-resistant CEM-

Table 1. Characteristics of the T- ALL cell lines included in this study

	Cell line			
Characteristics	**CEM-C7**	**CEM-C1**	**Jurkat**	**MOLT-4**
IC50 DEX, μM	0.8	>10	> 10	10
IC50 PRED, μM	1.8	>100	> 100	> 100
ATO, μM	2.0	1.8	1.2	0.76
GR status	*L753F het*	*L753F het*	*R477H het*	?
p53 status	het mut	het mut	het mut	no mutation
Nutlin assay	no effect	no effect	no effect	inhibition

IC50s were calculated using Graphpad Prism software with triplicate values from MTT assays. The GR mutation R477H was also found in families with generalized resistance to GC, mutation L753F was found in the patient samples from which the CEM line was derived, and probably is not relevant for GC-resistance in CEM-C1. p53 mutations as reported in the literature. We determined the dose response curves for nutlin-3 with the MTT assay.

het indicates heterozygous; mut, mutation; DEX, dexamethasone; and PRED, prednisone.

? indicates mutation status unknown.

C1, MOLT-4, and Jurkat cell lines were sensitive to ATO with IC50 values of 2.0, 1.8, 1.2, and 0.76 μM, respectively. This concentration range was safely achieved in the peripheral blood of adult APL patients.[33] Next, we performed combination experiments to evaluate the sensitizing effect of ATO on dexamethasone. A concentration of 0.25 μM ATO was sufficient to resensitize all 3 GC-resistant lines (CEM-C1, Jurkat, and MOLT-4) to dexamethasone (Figure 1A). At this concentration, ATO alone induced cell death in less than 5% of cells compared with untreated controls. In contrast, the response to dexamethasone could be augmented only slightly in GC-sensitive CEM-C7 cells (Figure 1A). We verified that increased cell death was indeed due to apoptosis induction by using flow cytometry to detect increased phosphatidylserine exposure and caspase activation in apoptotic cells (Figure 1B,C). Single-agent treatment with dexamethasone resulted in increased annexin-V positivity and caspase activation in the GC-sensitive CEM-C7 cells, but only slightly if at all in GC-resistant cells. Combination treatment of dexamethasone with 0.25 μM ATO increased annexin-V positivity and caspase activation compared with controls with either ATO alone or dexamethasone alone in GC-resistant cells, and to a lesser extent in GC-sensitive cells (CEM-C7). The amount of PI-positive cells was consistently low (between 0.3% and 4.5% of all the cells) with the exception of CEM-C1 cells treated with the combination of ATO and Dex, where 11.5% ± 0.85% of the cells were PI positive (Table S2). To verify that the effect of ATO was independent of the p53 status in ALL cell lines, we used the MDM2 antagonist nutlin-3, which induces apoptosis by displacing p53 from MDM2 only when p53 function is intact[34] (Table 1; Figure S2, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We found GC-resistant T-ALL cells that are responsive to ATO with (CEM, Jurkat) and without (MOLT-4) functional evidence for a p53 mutation. We also evaluated the effect of ATO in combination with agents that are commonly used for ALL induction chemotherapy. All 4 T-ALL cell lines were sensitive to asparaginase, vincristine, and daunorubicin and their drug-response profile was not altered by combination with ATO (Figure S1). To evaluate if glucocorticoid receptor (GR)–mediated transactivation could also contribute to GC sensitization by ATO, we performed combinatorial treatment experiments after transfection of a GR-responsive luciferase reporter (Figure S3). As expected, dexamethasone treatment resulted in increased transactivation of the GR-responsive reporter gene in GC-resistant cells, indicating the presence of functional GR in these cells. ATO did not influence reporter transactivation, either alone or in combination with dexamethasone. These results indicate that low-dose ATO can resensitize GC-resistant ALL cells to dexamethasone via GR-independent mechanisms.

Low-dose ATO generates oxidative stress and, in combination with dexamethasone, a reduction in Akt phosphorylation with concomitant effects on Akt targets Bad and XIAP

Given that induction of intracellular ROSs is one of the putative mechanisms of ATO-induced apoptosis in cancer cells, we determined the impact of different ATO concentrations on ROS generation (Figure 2A). Cells were treated with ATO concentrations of 0.25, 1, and 5 μM, and ROS production was measured by fluorometry. In GC-resistant cell lines, a 5-fold increase of ROSs was detected both at subtoxic (0.25 μM) and cytotoxic (1-5 μM) ATO concentrations. Since increased oxidative stress is commonly associated with activated oncogenic signaling, we focused next on the evaluation of key regulators of survival pathways. We first evaluated Akt, based on reports of high levels of phospho-Akt in lymphoblastic cell lines. Exposure to 1 μM ATO led to a decrease of phospho-ser 473 Akt, the activated phosphorylated form of Akt, within 10 hours of treatment, whereas the overall Akt levels remained unchanged (Figure 2B). We next focused on known Akt targets, including Bad, a proapoptotic member of the Bcl-2 family of intrinsic apoptosis pathway regulators,[18] and the X-linked inhibitor of apoptosis protein XIAP, a potent inhibitor of caspases.[19] Exposure to 1 μM ATO correlated with an increase in Bad protein levels after 16 hours of treatment. Simultaneously, XIAP levels were considerably decreased.

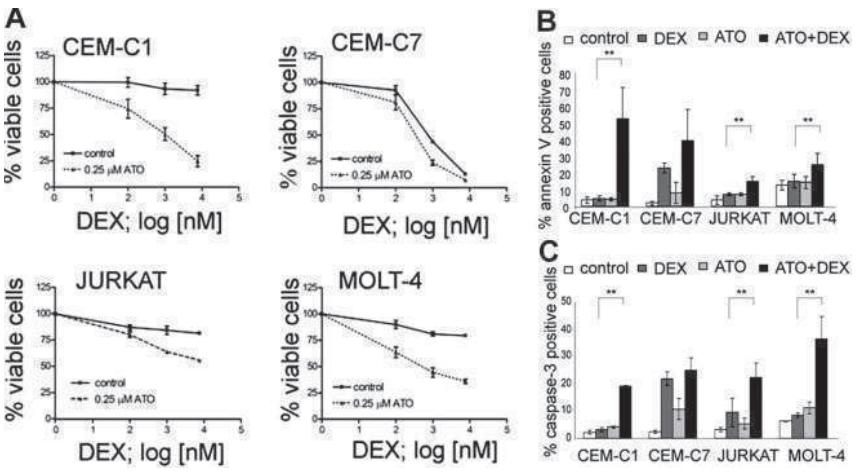

Figure 1. ATO sensitizes resistant T-ALL cell lines to dexamethasone treatment. (A) Response to increasing concentrations of dexamethasone (DEX) after 72-hour incubation with or without 0.25 μM ATO. Cell viability was assessed using the MTT assay. (B) Apoptosis rates were determined using annexin-V/propidium iodide stainings. Cells were incubated for 48 hours (CEM-C1, CEM-C7, and MOLT-4) or 72 hours (Jurkat) with control, 0.25 μM ATO, 7.6 μM Dex (or 0.1 μM for CEM-C7), and the appropriate combination and analyzed by flow cytometry. (C) Induction of caspase-3 activity was assessed by flow cytometry. For panel A, the measurements were normalized to untreated controls, and for all experiments, values of 3 experiments are shown as mean plus or minus SD; $^{**}P < .05$ (Student *t* test).

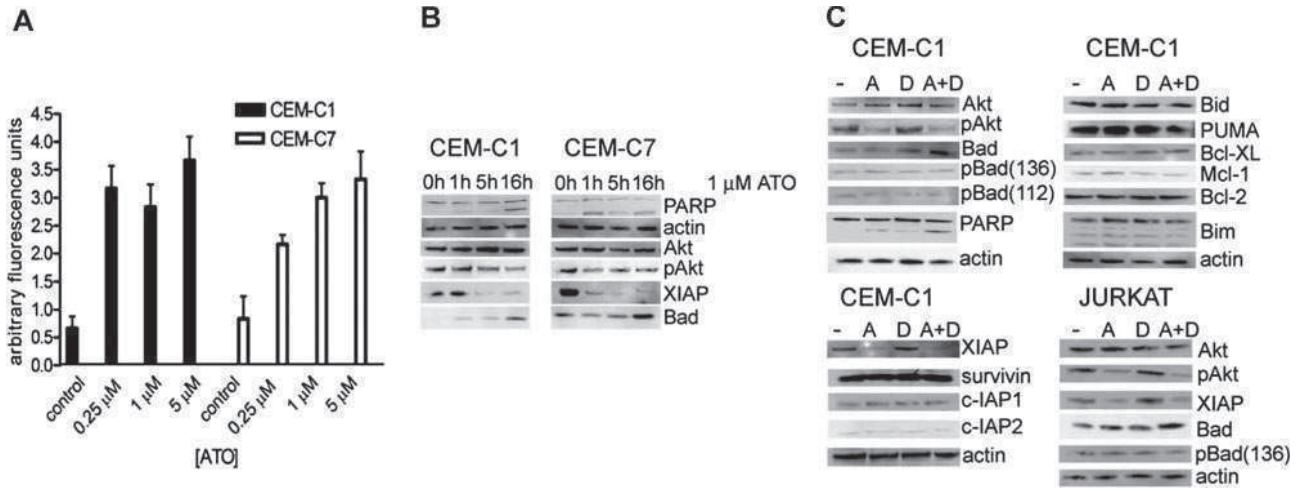

Figure 2. ATO treatment induces ROS production and dephosphorylation of Akt, which is associated with an increase of Bad and decrease of XIAP. (A) ROS generation in CEM-C1 and -C7 was quantified by fluorometry with varying concentrations of ATO as indicated. Values of 3 experiments are shown as mean (−SD); *P* equals .05 (Student *t* test). (B) ATO as single agent. CEM-C1 and -C7 cells were incubated for different times with 1 μM ATO. Western blot analysis using whole-cell extracts from a representative experiment is shown. Antiactin was used for loading control. (C) Combination for 5 hours of 0.25 μM ATO with 1 μM DEX in CEM-C1 and Jurkat cells; A indicates ATO; D, DEX.

If modulation of Akt, Bad, and XIAP were relevant for the synergistic effect observed with low-dose ATO and dexamethasone, then the combination treatment should have similar effects on these targets as ATO alone. Combination treatment resulted in a decrease of Akt phosphorylation and in an increase of Bad compared with low-dose ATO treatment and similar to the effect obtained with 1 μM ATO alone (Figure 2C). Detection of PARP cleavage as a measure of apoptosis appeared more pronounced in the combination treatment when compared with the single agents. A sharp decrease of XIAP levels occurred with 0.25 μM ATO, without detectable difference when dexamethasone was added. Also included in our panel were a number of proapoptotic and antiapoptotic regulators. As shown in Figure 2C, levels of the proapoptotic BH3-only proteins PUMA, Bim, and Bid as well as the antiapoptotic regulators Bcl-2, Bcl-XL, and Mcl-1 were unchanged after exposure to ATO and dexamethasone. These results indicate that inhibition of Akt is a possible mechanism of the sensitizing effect of low-dose ATO on dexamethasone. Modulation of Bad and XIAP may be relevant for this effect, although the effect on XIAP could also be mediated by another ATO-dependent mechanism. To verify whether other IAP family members were affected, we analyzed the expression levels of survivin, c-IAP1, and c-IAP2 after treatment with either ATO alone, dexamethasone alone, or the combination of both. As shown in Figure 2C, expression levels of these IAP family proteins were unchanged suggesting that down-regulation of XIAP is a specific effect.

Transient expression of dominant-active myrAkt decreases GC response in sensitive cells and interferes with the GC-sensitizing effect of ATO

To verify the relevance of Akt inactivation for the GC-sensitizing effect of ATO, we used a dominant-active myristoylated form of Akt.[31] We transiently expressed myrAkt for 24 hours in CEM-C1 cells and analyzed the effect on cell viability following treatment with dexamethasone or the combination of ATO at 0.25 μM and varying concentrations of dexamethasone of 0.1, 1, and 7.6 μM for 48 hours. Transient expression of myrAkt conferred complete resistance to dexamethasone treatment and inhibited the sensitizing effect of ATO to dexamethasone, rendering CEM-C1 resistant to the combination treatment (Figure 3A). One would expect that myrAkt expression would also alter the expression of the Akt targets XIAP and Bad in this experiment. Indeed, myrAkt expression rescued XIAP protein levels, and Bad phosphorylation was increased compared with mock-transfected cells (Figure 3B). XIAP down-regulation was inhibited and Bad phosphorylation at Ser136 was maintained at control levels when myrAkt-overexpressing cells were incubated with the combination of ATO and dexamethasone. These data indicate that Akt activity protects XIAP from degradation induced by low-dose ATO and dexamethasone as well as maintains Bad in its inactive phosphorylated state.

Since the members of the IAP family are transcriptionally regulated by NF-κB,[35] we also evaluated the effect of ATO on NF-κB activation. Cells were stimulated with ATO, dexamethasone, or in combination, and the effect on nuclear translocation of NF-κB was analyzed. We could not detect any decrease in the nuclear translocation of the p65 subunit of NF-κB in this experiment (Figure S3). Collectively, our results suggest that a pathway involving Akt, XIAP, and Bad is affected as a result of exposure to ATO and dexamethasone.

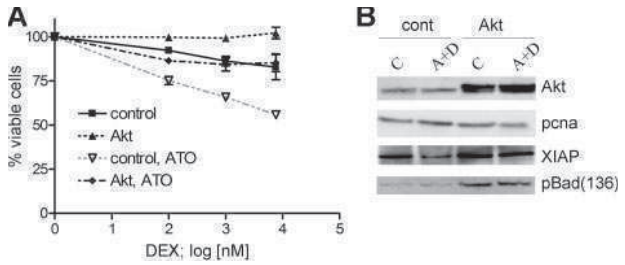

Figure 3. Transient expression of myrAkt impairs the sensitizing effect of ATO on dexamethasone-mediated cell death. (A) Dominant-active myrAkt was transiently expressed in CEM-C1 cells. MyrAkt rendered cells completely resistant to dexamethasone treatment and reduced the effect of 0.25 μM ATO on the cytotoxic effect of increasing DEX concentrations (0.1 μM, 1.0 μM, 7.6 μM). Values from triplicate MTT assays are shown as mean plus or minus SD. (B) myrAkt expression in CEM-C1 cells enhances the levels of Ser136 p-Bad and restores XIAP. Cells were treated as indicated 24 hours after transfection. Whole-cell extracts were obtained after 5 hours of drug exposure and analyzed by Western blot.

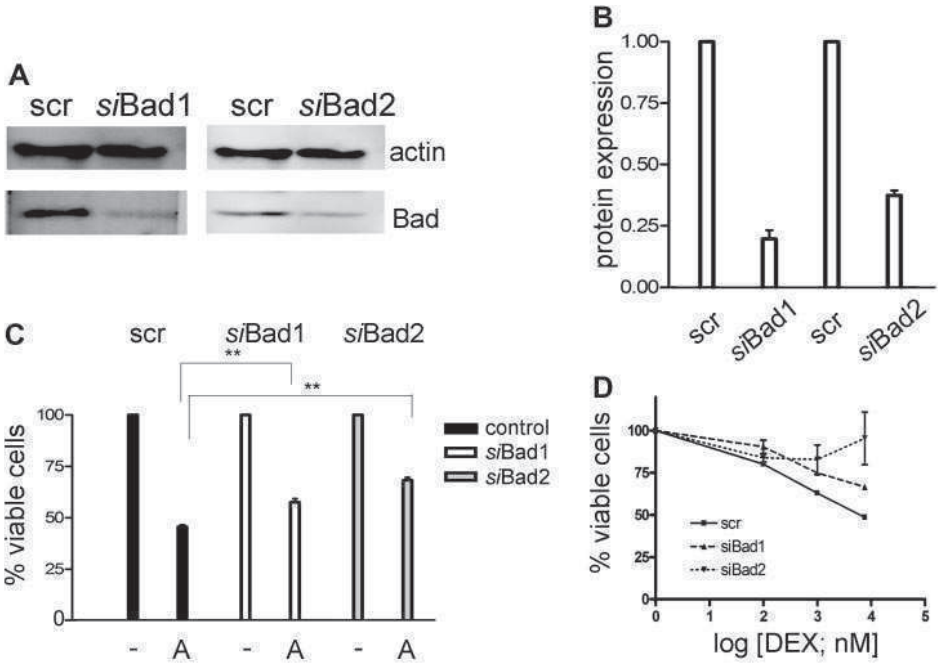

Figure 4. Down-regulation of Bad using siRNA decreases the sensitivity of the cells to the combination treatment of ATO and dexamethasone. (A) CEM-C1 cells were transfected with 2 distinct siRNAs directed against Bad. Down-regulation of Bad was assessed after 48 hours by Western blot. (B) Signal quantification by densitometry. (C) *si*Bad treatment of CEM cells decreased the cytotoxic effect of 1 μM ATO as single agent and (D) of the combination of low-dose ATO (0.25 μM) with DEX. A scrambled siRNA was used for control. Triplicate results of the MTT assay are shown as mean plus or minus SD; $^{**}P < .05$ (Student *t* test).

Down-regulation of Bad and overexpression of XIAP confer resistance to ATO-induced GC sensitization

To demonstrate that inactivation of Bad may play a role in GC resistance, we knocked down Bad levels by RNA interference in CEM-C1 cells (Figure 4A). A significant decrease of Bad was observed with 2 different specific siRNAs, but not with a scrambled control (Figure 4B), which correlated with a reduction of ATO-mediated cytotoxicity at 1-μM concentration (Figure 4C). As expected, down-regulation of Bad affected the sensitizing effect of low-dose ATO in combination with dexamethasone, whereby one of the specific siRNAs almost completely rescued the GC-resistance phenotype (Figure 4D). These results are consistent with the modulation of a pathway involving Bad in GC sensitization, but imply that additional mechanisms account for the full effect of ATO treatment.

Rapid down-regulation of XIAP due to loss of the stabilizing phosphorylation on serine residue 87 could represent such a mechanism. We therefore investigated the effect of XIAP overexpression on dexamethasone sensitization. To evaluate the relevance of the Akt phosphorylation site, we mutated serine 87 to either glutamine (XIAP-S87E, gain of function) or alanine (XIAP-S87A, loss of function) as reported previously.[19] As anticipated, overexpression of XIAP rendered CEM-C1 cells resistant to ATO-induced GC-mediated cell death (Figure 5A). Despite overexpression levels at 25% of wild-type XIAP, XIAP-S87E induced GC resistance to a similar extent (Figure 5B). In contrast, XIAP-S87A expression was clearly less effective at rescuing cells from ATO-induced GC-sensitization at expression levels that were comparable to the S87E mutant. The correlation of decreased XIAP levels with inhibition of Akt phosphorylation could be explained by an effect on XIAP protein turnover. Indeed, the dominant-active mutant XIAP-S87E, which is less susceptible to proteasomal mediated degradation, was able to restore the resistance phenotype at much lower expression levels compared with the wild-type protein (Figure 5A,B). Collectively, our results show that dephosphorylation of Bad and destabilization of XIAP levels, as a consequence of Akt inhibition, are important for the GC-sensitizing effect of ATO.

Combination of ATO with the mTOR inhibitor rapamycin is not synergistic

Having established Akt as important mediator in the GC sensitization, and given that mTOR is also a potential target of Akt, we asked whether intrapathway inhibition of Akt and mTOR by combination of rapamycin with ATO increases the GC sensitization effect of ATO (Figure S4). As reported recently,[21] rapamycin augmented the GC sensitivity of resistant CEM cells, an effect that was shown to be associated with decreased Mcl-1 levels. Indeed, 10 nM rapamycin sensitized CEM-C1 to dexamethasone with a comparable efficiency with the results achieved with 0.25 μM ATO. However, combination of the 2 agents was not synergistic. Thus, the 2 approaches appear to be equivalent but not complementary in the CEM cell-line model of GC resistance.

ATO improves glucocorticoid sensitivity of primary leukemic cells from ALL patients with poor in vivo response to prednisone

In order to validate the results obtained in cell lines, we selected pretreatment samples from 7 ALL patients at disease presentation

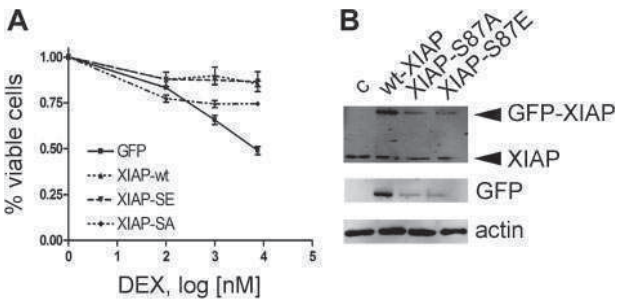

Figure 5. Resistance to ATO-mediated GC sensitization by XIAP overexpression requires the XIAP-S87 phosphorylation site. (A) Response to increasing concentrations of DEX after 72-hour incubation in combination with 0.25 μM ATO, after transient transfection of different XIAP constructs as indicated and described in "Patients, materials, and methods." Triplicate results of the MTT assay are shown as mean plus or minus SD. (B) Western blot with specific anti-XIAP antibody (top panel) and an anti-GFP antibody (bottom panel).

Table 2. Patients characteristics

Characteristic	Patient no.						
	1	2	3	4	5	6	7
Age, y	9	2	13	13	3	3	2
Subtype	T	T	pc-B	pc-B	T	pc-B	pc-B
Blast, d0	207	338	70	69	34	0.1	69
Blast, d8	127	1.8	5.7	1.8	0.7	0	0
Pred. resp*	poor	poor	poor	poor	good	good	good
BM blast d0	92%	95%	n/a	99%	87%	98%	96%
MRD†	HR	non-HR	HR	non-HR	non-HR	non-HR	non-HR

Blast in Giga/L

T indicates T-ALL; pc-B, precursor-B-ALL; Pred. resp, prednisone response; BM, bone marrow; HR, high risk and n/a, not available.

* Prednisone poor response is defined as more then 1000 blasts/μL at day 8 of prednisone therapy.

†HR MRD is defined as an MRD load of 10^{-3} or greater at week 12 of treatment.

based on their prednisone response.[1] We included 2 patients with poor initial response to prednisone (PPR) and persistence of minimal residual disease after induction chemotherapy (MRD-HR), 2 patients with a PPR but low or no detectable MRD at week 12 (non–MRD-HR), and 3 patients with good response to prednisone (PGR) (Table 2). We first determined the response to ATO as a single agent in vitro (Figure 6A). At low concentration, ATO was not cytotoxic for normal bone marrow leukocytes. The IC50 values for ATO-sensitive leukemia cells (3 PPR, 1 PGR) were comparable with the responses obtained in cell lines. Low-dose ATO treatment clearly increased the dexamethasone sensitivity of T-ALL and pre-B-ALL cells from prednisone poor-responder patients (Figure 6B, Pt 1-4), but not in cells obtained from the prednisone good-responder patients (Figure 6B, Pt 5-7). This response was observed in both T-ALL (Pt 1-2) and precursor B-ALL (Pt 3-4), similar to the observations made in cell lines. These results indicate that ATO sensitization to dexamethasone does occur in primary cells from GC-resistant patients and suggest that combination therapy with ATO might be beneficial for GC-resistant patients.

Discussion

We report here that subtoxic doses of arsenic trioxide (ATO) can restore the sensitivity to dexamethasone in glucocorticoid (GC)–resistant T and precursor B acute lymphoblastic leukemia cells and provide evidence for the Akt/XIAP pathway as candidate target for combinatorial therapy in GC resistance.

Characteristic profiles of activated survival signals can be detected in leukemia.[36,37] One of the key signaling nodes that is commonly activated in human tumors is Akt.[14] High levels of endogenous phospho-Akt in ALL cell lines correlated with an increased sensitivity to the PI3K/Akt inhibitor LY294002,[17] suggesting that this pathway may be important for the survival of leukemia cells. Constitutive activation of prosurvival pathways could contribute to drug resistance by promoting the resistance to apoptosis. Recent gene expression profiling data suggest that Akt might indeed play a role in GC-resistant ALL. Gene set enrichment analysis revealed that among gene sets derived from the BioCarta pathway database (San Diego, CA), the Akt pathway was the

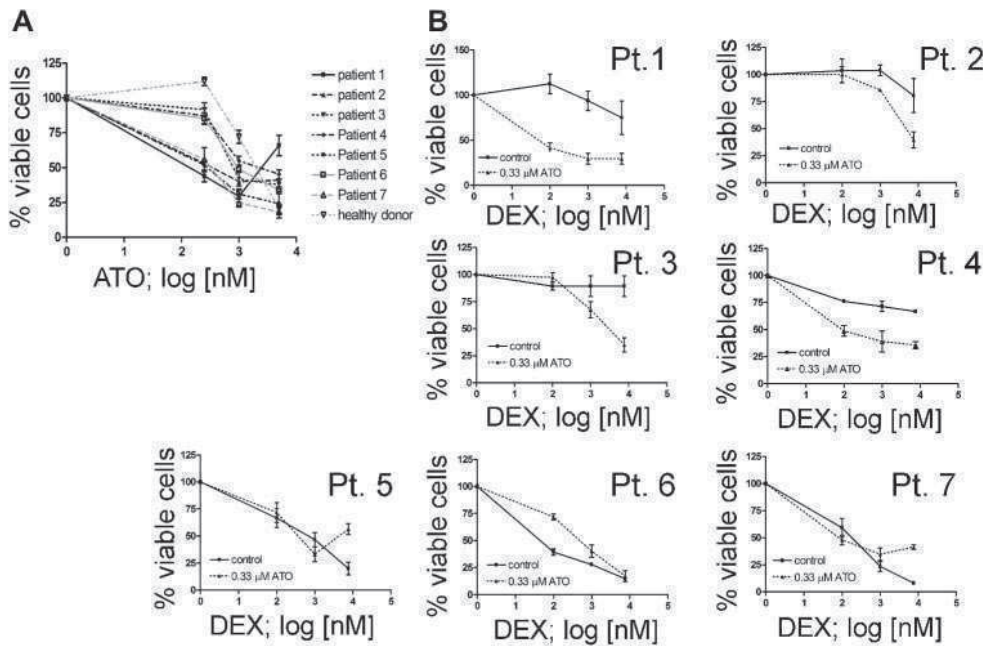

Figure 6. ATO sensitizes primary leukemia cells from prednisone poor-responder patients to dexamethasone. (A) Effect of ATO as single agent on 7 ALL patients who are described in Table 2. No cytotoxic effect could be detected in bone marrow leukocytes from a healthy donor at 0.33 μM ATO. (B) Response to increasing concentrations of DEX with or without 0.33 μM ATO on ALL cells from prednisone poor responders with T-ALL (Pt 1-2), pre-B-ALL (Pt 3-4), from prednisone good responders with T-ALL (Pt 5) and pre-B-ALL (Pt 6-7).

most highly enriched set in GC-resistant pre-B-ALL samples.[21] Because different oncogenic events may result in activation of the Akt pathway,[14] approaches to target this pathway may be more widely applicable and not dependent on the presence of a particular mutation. Based on these considerations, we postulated that the broad range of biologic activities of ATO, including its potential to inhibit the Akt pathway,[15] could offer an advantage in combination therapy of GC-resistant ALL.

The recent report that single-agent ATO treatment did not induce a clinical response in 11 adult patients with relapsed and refractory ALL[38] should not preclude further evaluation of this agent in combination therapy in ALL. For example, combination of ATO with the tyrosine kinase inhibitor imatinib resulted in more efficient induction of apoptosis in chronic myelogenous leukemia (CML).[39] We have shown here that subtoxic concentrations of ATO (0.25 μM) increase the sensitivity to dexamethasone effectively in GC-resistant cells, which is reflected by a level of ROS production comparable with levels achieved at higher cytotoxic ATO doses (1-5 μM). This is relevant because toxicity on normal hematopoietic tissues was not relevant using ATO concentrations below 0.5 μM.[40,41] Thus, lower doses of ATO may be sufficient to achieve clinically relevant drug-sensitization effects.

Different pathways were altered by ATO treatment depending on the type of hematologic malignancy studied, such as the MAPK pathway in AML cells and the NF-κB pathway in myelodysplastic syndrome.[30,42] This may reflect context-dependent differences in signal transduction pathway activation and illustrates the potential advantage to use an agent with such a broad target range. We found evidence mostly for an implication of Akt and downstream Akt targets in GC sensitization by ATO, but recognize that we have examined only a subset of downstream effectors that have been previously implicated in the response to ATO.[28] Among other possible mechanisms that we evaluated, we could not detect changes in Erk phosphorylation or find evidence for NF-κB inhibition. In our hands, ATO treatment also did not result in nuclear translocation of the apoptosis-inducing factor (AIF) (data not shown).

Our results show that ATO inhibits Akt activation and influences 2 important regulators of apoptosis, the proapoptotic Bcl-2 family member Bad and a member of the inhibitor of apoptosis protein (IAP) family, XIAP. Down-regulation of Bad by RNA interference decreased ATO-mediated GC sensitivity suggesting that part of the ATO effect is mediated through interference with the intrinsic apoptotic pathway via Bad. We also provide data for a complementary mechanism at the postmitochondrial level via regulation of XIAP, which strongly regulates apoptosis by direct binding and inactivation of the caspases-3, -7, and -9. XIAP was reported to be stabilized by Akt phosphorylation, resulting in a decrease of ubiquitin-mediated protein degradation.[19] Consistent with the hypothesis that Akt inhibition could influence XIAP protein stability, we observed a sharp decrease of XIAP after ATO treatment. Accordingly, overexpression of dominant-active myrAkt restored the XIAP levels, which correlated with GC resistance. Furthermore, the presence of the Akt phosphorylation site on XIAP was required for a complete reversion of the GC sensitization by ATO. Down-regulation of XIAP levels could also be due in part to inhibition of translation resulting from a decrease of mTOR activity, since mTOR is a target of Akt and a decrease of XIAP translation was reported as part of the synergistic effect of rapamycin and dexamethasone combination in multiple myeloma.[22] However, this would not explain why other IAPs, survivin, cIAP1, and cIAP2, were not affected by ATO treatment. Nevertheless, our experiments identified the Bcl-2 family member Bad and XIAP as candidate targets for combination therapy in drug-resistant ALL and provide a rationale for the evaluation of more specific small molecule inhibitors that target apoptotic regulators in GC-resistant ALL.[43,44]

Given the crosstalk between mTOR and Akt,[45] the ATO effect could also be mediated via Akt-induced mTOR inhibition. Indeed, recent data show that the mTOR inhibitor rapamycin increased the sensitivity of CCRF-CEM cells to GC.[21] This effect was associated with down-regulation of the antiapoptotic regulator Mcl-1. However, we could not detect any effect of ATO-mediated Akt inhibition on Mcl-1 levels, implying that either indirect inhibition of mTOR was not relevant for the ATO effect or that Mcl-1 down-regulation may not be a direct effect of mTOR inhibition. The combination of ATO and rapamycin did not increase the sensitivity of CEM cells to GC. This suggests that the 2 agents could have overlapping effects. However, rapamycin was shown to revert the resistance phenotype conferred by myrAkt overexpression in GC-sensitive lymphoid cells only partially,[21] indicating that part of the resistance mechanism is independent of mTOR signaling. Our results also indicate that down-regulation of Mcl-1 is not essential for GC sensitization, supporting the concept that Mcl-1–independent mechanisms are important. Among other Bcl-2 family members that could be involved in GC resistance, a failure of Bim induction has been described to occur in GC-resistant ALL cells.[2] In our experiments, an increase in Bim isoform levels was not detected in ATO-mediated GC sensitization.

Experiments with primary cells are indispensable to model drug resistance in pre-B-ALL, due to the lack of representative cell lines. We did detect a GC-sensitizing effect using low-dose ATO consistently in GC-resistant samples, but not in GC-sensitive samples, both from T-ALL and precursor B-ALL patients. This observation strongly suggests that GC-resistant ALL cells may be addicted to prosurvival pathways that can be influenced by ATO treatment. Improved preclinical models of de novo drug-resistant ALL will be required in order to define the profiles of pathway activation that may predict resistance to different drugs in MRD-HR ALL and to test appropriate agents in combination therapy. Our data indicate that combination of low-dose ATO with glucocorticoids may be a valuable and affordable approach to improve the treatment of GC-resistant ALL.

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Authorship

Contribution: B.C.B., B.W.S., and J.-P.B. designed the experiments and analyzed the data; B.C.B, L.B., and R.M. performed the experiments; J.-P.B. drafted and wrote the paper; F.K.N, G.C., M.S., D.L., and R.M. contributed essential reagents.

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Correspondence: Jean-Pierre Bourquin, Department of Oncology, University Childrens Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich; e-mail: jean-pierre.bourquin@kispi.uzh.ch.

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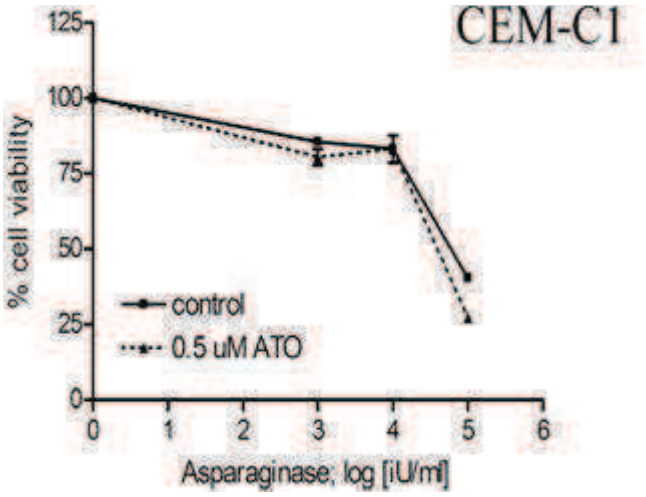
	control	ATO	Dex	ATO+Dex
CEM-C1	0.45±0.17	0.45±0.03	1.45±0.53	11.5±0.85
CEM-C7	0.3±0.14	0.75±0.53	3.45±2.01	4.45±0.67
MOLT-4	1.67±0.17	0.97±0.17	1.9±0.07	1.97±0.17
JURKAT	0.5±0.07	0.8±0.42	0.65±0.32	0.75±0.25

Note: the number of PI positive cells were determined as described under material and methods.


CEM-C1
% cell viability
125
100
75
50
25
0
control
0.5 uM ATO
0
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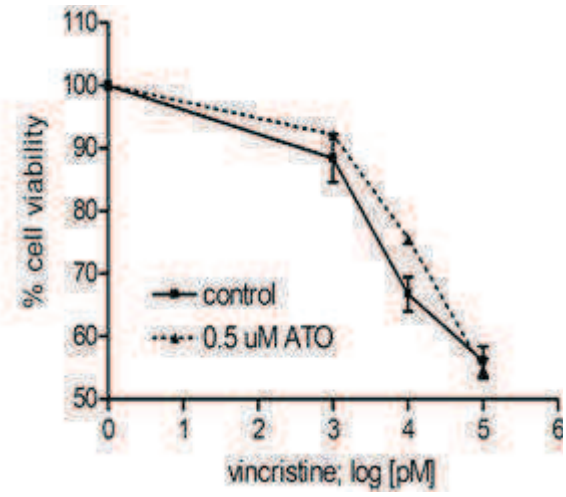
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110
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0
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vincristine; log [pM]

Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance

Laura Bonapace,[1] Beat C. Bornhauser,[1] Maike Schmitz,[1] Gunnar Cario,[2] Urs Ziegler,[3] Felix K. Niggli,[1] Beat W. Schäfer,[1] Martin Schrappe,[2] Martin Stanulla,[2] and Jean-Pierre Bourquin[1]

[1]Department of Oncology, University Children's Hospital, University of Zurich, Switzerland. [2]Department of Pediatrics, University Hospital Schleswig Holstein, Kiel, Germany. [3]Center for Microscopy and Image Analysis, University of Zurich.

In vivo resistance to first-line chemotherapy, including to glucocorticoids, is a strong predictor of poor outcome in children with acute lymphoblastic leukemia (ALL). Modulation of cell death regulators represents an attractive strategy for subverting such drug resistance. Here we report complete resensitization of multidrug-resistant childhood ALL cells to glucocorticoids and other cytotoxic agents with subcytotoxic concentrations of obatoclax, a putative antagonist of BCL-2 family members. The reversal of glucocorticoid resistance occurred through rapid activation of autophagy-dependent necroptosis, which bypassed the block in mitochondrial apoptosis. This effect was associated with dissociation of the autophagy inducer beclin-1 from the antiapoptotic BCL-2 family member myeloid cell leukemia sequence 1 (MCL-1) and with a marked decrease in mammalian target of rapamycin (mTOR) activity. Consistent with a protective role for mTOR in glucocorticoid resistance in childhood ALL, combination of rapamycin with the glucocorticoid dexamethasone triggered autophagy-dependent cell death, with characteristic features of necroptosis. Execution of cell death, but not induction of autophagy, was strictly dependent on expression of receptor-interacting protein (RIP-1) kinase and cylindromatosis (turban tumor syndrome) (CYLD), two key regulators of necroptosis. Accordingly, both inhibition of RIP-1 and interference with CYLD restored glucocorticoid resistance completely. Together with evidence for a chemosensitizing activity of obatoclax in vivo, our data provide a compelling rationale for clinical translation of this pharmacological approach into treatments for patients with refractory ALL.

Introduction

Resistance to the initial phase of chemotherapy, in particular poor response to glucocorticoids (GCs), is a strong predictor of adverse outcome for childhood acute lymphoblastic leukemia (ALL) (1, 2). During the last decade, the cooperative Berlin-Frankfurt-Muenster (BFM) study group has validated an effective risk stratification approach, which is based on the assessment of the in vivo response to chemotherapy by leukemia-specific quantitative PCR. A group of patients at very high risk for relapse (VHR-ALL) can be identified based on persistence of minimal residual disease (MRD) (3). Because this is likely to reflect de novo resistance to multiple conventional antileukemic agents, combination treatment with new agents that modulate regulators of cell death represents an attractive approach to improve treatment response.

In GC-resistant ALL, the mechanisms underlying defective induction of mitochondrial apoptosis are still not understood. Increased expression of antiapoptotic myeloid cell leukemia sequence 1 (MCL-1) was a predominant feature of the gene expression signature of GC resistance (4). A bioinformatic screen of drug-associated signatures identified rapamycin as a sensitizer to GC drugs in GC-resistant ALL. The GC-sensitizing effect of rapamycin was attributed to a decrease in MCL-1 levels, which was proposed to decrease the threshold to apoptotic stimuli by GC drugs (5).

Based on these considerations, we sought to evaluate the potential of the small molecule obatoclax (GX15-070) for combination therapy in refractory childhood ALL. This agent was proposed to act as a BCL-2 family antagonist and to disrupt the interaction between MCL-1 and its proapoptotic counterparts at cytotoxic concentrations (6, 7). Obatoclax was shown to trigger apoptosis at concentrations that resulted in disruption of Bak from MCL-1 and cytochrome *c* release. However, obatoclax was also cytotoxic in cells that are deficient for the apoptosis effectors BAX and BAK, and lower concentrations of compound were sufficient to inhibit clonogenic growth of AML cells, suggesting the existence of additional target mechanisms (8). A recommended phase II dose has been established for adult patients with hematologic malignancies with an acceptable toxicity profile (9, 10), which constitutes the basis for further evaluation of obatoclax in pediatric trials.

In the context of defective apoptosis, an alternative cell death pathway has been identified that is dependent on macroautophagy (11, 12), a major form of autophagy, in which parts of the cytoplasm and intracellular organelles are sequestered within characteristic double-membraned or multi-membraned autophagic vacuoles (hereafter referred to as autophagy) (13). Autophagy is usually triggered to respond to increased metabolic requirements at times of cellular stress. Selected antiapoptotic BCL-2

Authorship note: Laura Bonapace and Beat C. Bornhauser contributed equally to this work.

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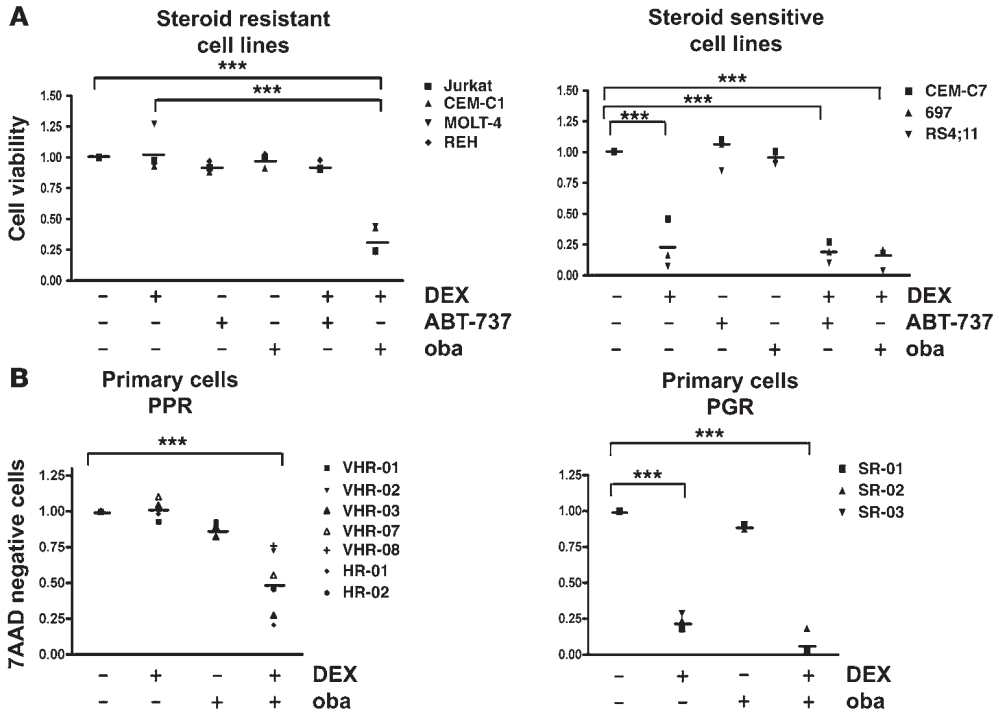


Figure 1

Obatoclax resensitizes GC-resistant ALL cells to dexamethasone. Combination experiments were performed with subcytotoxic concentrations (10% IC_{50}) of obatoclax (oba) or ABT-737 and 1 μM dexamethasone (DEX), and values were normalized to cells treated with vehicle control. (**A**) ALL cell lines were treated for 72 hours as indicated. Cell viability was determined by the MTT assay. (**B**) Primary ALL cells from 7 PPR patients with VHR-ALL and 3 prednisone-good-responder (PGR) patients were treated as indicated for 72 hours. Cell viability was assessed by flow cytometry using 7AAD. ***$P < 0.05$.

family members can engage in cross-talk between the apoptotic and autophagic pathways, as they were show to associate with the autophagy regulator beclin-1 (11, 14, 15). When caspase-dependent apoptosis was blocked, a cell death mechanism that required autophagy was mediated via the receptor-interacting protein 1 (RIP-1) (12). RIP-1 is a central kinase that is associated with death receptor–induced signalling complexes to modulate the switch between survival and death under stress conditions (16). Under defective apoptotic conditions, RIP-1 kinase activity was shown to mediate an alternative cell death pathway that may represent a form of programmed necrosis, also called necroptosis (17, 18). Kinase activity of RIP-1 was shown to be dispensable for induction of cell survival signalling via NF-kB activation or death receptor–mediated apoptosis (17, 19).

Here we show that a subcytotoxic concentration of obatoclax effectively restored the response to dexamethasone in GC-resistant ALL by triggering a nonapoptotic cell death pathway. Subcytotoxic concentrations of obatoclax induced disruption of beclin-1 from MCL-1 and the combination of dexamethasone with obatoclax was associated with inhibition of mammalian target of rapamycin (mTOR) activity, providing a possible mechanism for autophagy induction. Obatoclax also conferred clinically relevant broad chemosensitization in multidrug-resistant primary cells from VHR-ALL patients. We provide genetic and pharmacologic evidence to show that sensitization to dexamethasone occurs via autophagy-dependent necroptotic cell death, while sensitization to other cytotoxic agents was dependent on apoptosis. In a xenograft model, using GC-resistant cells from refractory ALL patients, combination treatment with dexamethasone and obatoclax reduced leukemia progression significantly. Collectively, our data provide a strong rationale for a rapid clinical translation of this approach.

Results

Obatoclax resensitizes GC-resistant ALL to dexamethasone. Because MCL-1 has been proposed to act as a central modulator of steroid resistance in ALL (4, 5), we first compared the effects of 2 small molecules with different selectivity toward MCL-1, ABT-737, and obatoclax in GC-resistant ALL cell lines. ABT-737 is a small molecule BH3 mimetic with selectivity for BCL-2 and BCL-X_L (20). Resistance to ABT-737 has been attributed to its inability to target MCL-1 (21–23). While both compounds displayed strong cytotoxicity across a panel of ALL cell lines (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI39987DS1), subcytotoxic doses of obatoclax but not ABT-737 resensitized GC-resistant cell lines to dexamethasone (Figure 1A and Supplemental Figure 1). We confirmed single-agent activity for obatoclax in VHR-ALL cells (Supplemental Table 2). Low-dose obatoclax restored sensitivity to dexamethasone in cells from all 7 poor-prednisone-responder (PPR) VHR-ALL patients tested (Figure 1B and Supplemental Figure 2). In contrast, no effect on steroid sensitivity using low doses of ABT-737 or obatoclax was evident in steroid-sensitive cells (Figure 1A). Unexpectedly, the pan-caspase inhibitor zVAD.fmk did not interfere with the steroid-sensitizing effect of obatoclax in GC-resistant cells (Figure 2A), whereas zVAD.fmk blocked the cytotoxic effect of dexamethasone with or without obatoclax in GC-sensi-

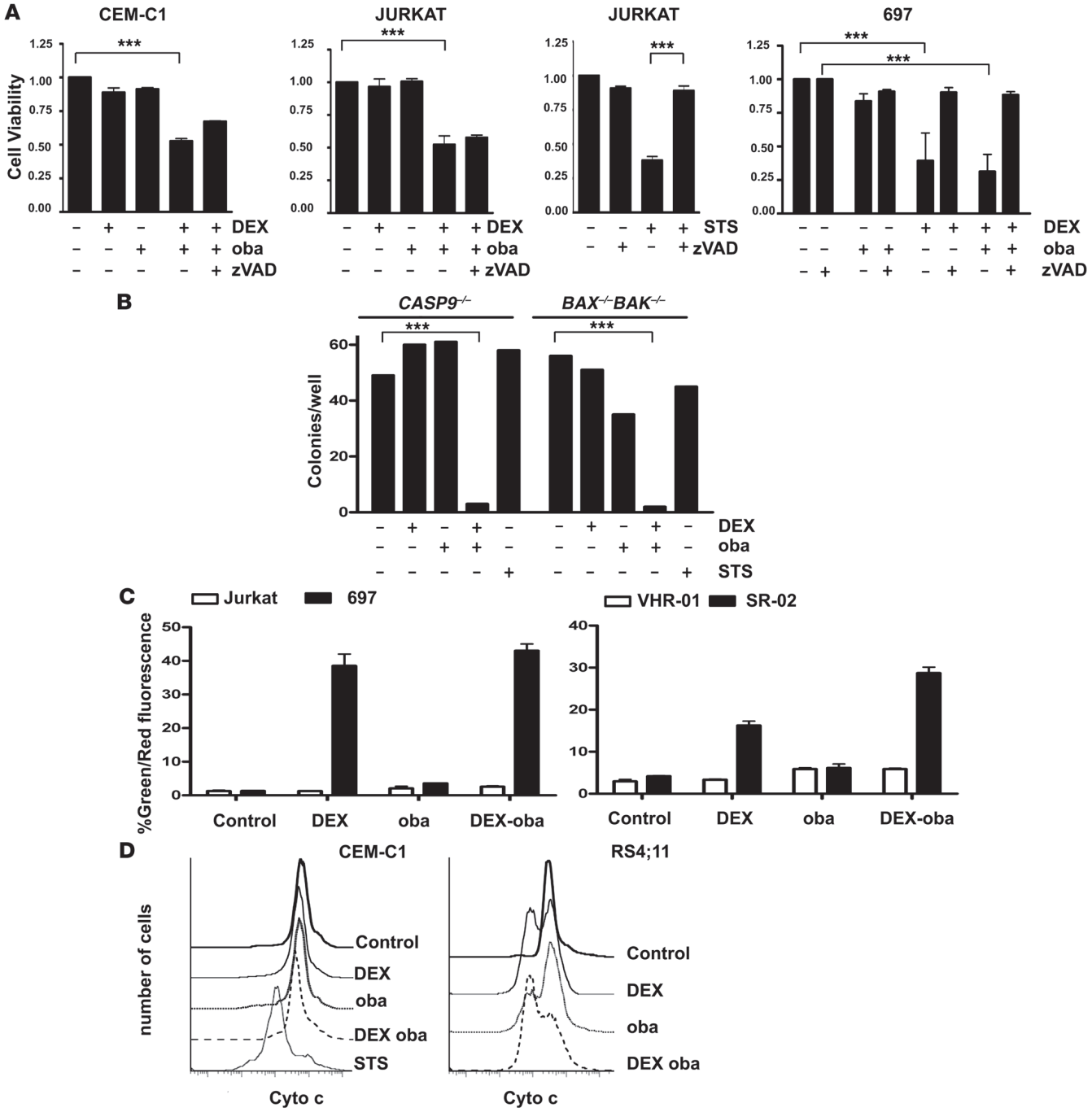


Figure 2

Obatoclax resensitizes GC-resistant ALL cells to dexamethasone without activation of mitochondrial apoptosis. (**A**) ALL cells were treated as indicated for 48 hours, for controls STS or zVAD.fmk (80 nM) was used, and cell viability was assessed with the MTT assay. 697 cells served as GC-sensitive control. (**B**) Jurkat *CASP9*$^{-/-}$ and *BAX*$^{-/-}$*BAK*$^{-/-}$ cells were treated for 72 hours as indicated, and clonogenic survival was assessed after incubation in methylcellulose for 7 days. (**C**) Percentages of cells with JC-1 monomers, corresponding to a loss of the mitochondrial potential, are shown for GC-resistant CEM-C1 and GC-sensitive CEM-C7 cells and in samples from PGR and PPR patients after treatment as indicated for 16 hours. (**D**) Cytochrome *c* release was induced in steroid-sensitive RS4;11 cells but not in the resistant CEM-C1 cell line upon treatment with dexamethasone or dexamethasone and obatoclax. STS was used as positive control. Cytochrome *c* release was detected by flow cytometry. ***$P < 0.05$.

tive cells and in control experiments when cell death was induced by staurosporine (STS). Using clonogenic assays, we confirmed that obatoclax was equally effective in ALL cells that are devoid of caspase-9 or BAX and BAK, when compared with parental cell lines (Figure 2B and Supplemental Figure 3). Furthermore, obatoclax-mediated GC sensitization resulted in neither the restoration of mitochondrial membrane depolarization (Figure 2C) nor an increase in cytochrome *c* release when compared with the effect

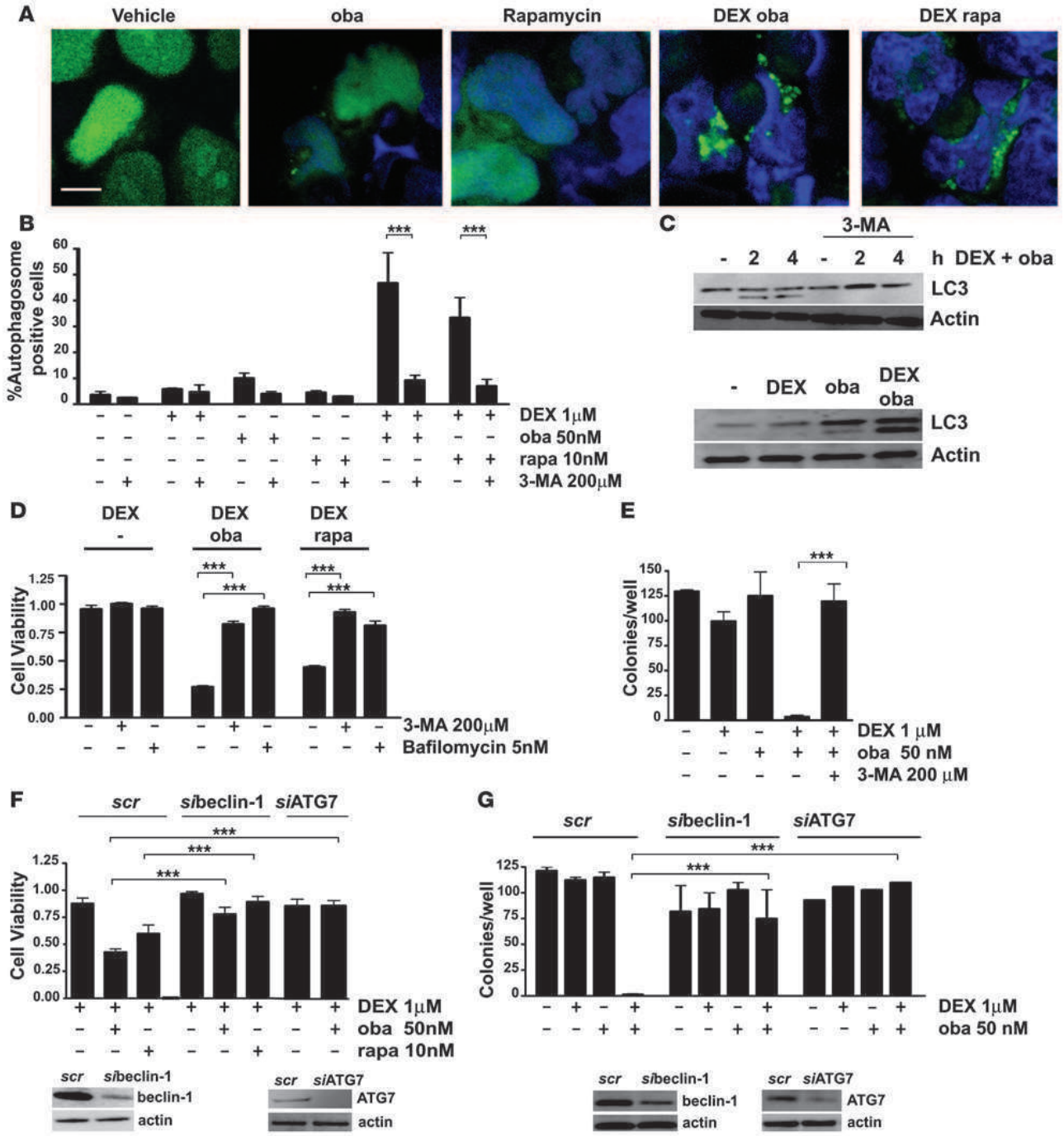


Figure 3

Obatoclax induces autophagy in GC-resistant ALL cells. (**A**) After transient transfection with GFP-LC3, Jurkat cells were treated for 4 hours as indicated. The characteristic punctuate staining pattern indicative of autophagosome formation was detected by confocal microscopy in cells treated with dexamethasone and obatoclax or rapamycin (rapa). Scale bar: 10 μm. (**B**) Quantitation of autophagosome-positive cells. The data represent mean ± SD of 2 independent experiments, counting 200 cells each. (**C**) Detection of endogenous LC3-II by Western blot analysis. Jurkat cells were treated with obatoclax and dexamethasone for the indicated time points in the presence or absence of 3-MA and after 24 hours of treatment as indicated. (**D**) Inhibition of autophagy by 3-MA or bafilomycin impaired sensitization to dexamethasone by 10 nM rapamycin or obatoclax (10% IC_{50}). Cell viability was assessed by MTT. (**E**) Treatment with obatoclax and dexamethasone inhibited clonogenic survival of Jurkat cells. 3-MA rescued GC-resistant cells from cell death induced by combination treatment. Cells were treated for 72 hours with compounds, and clonogenic survival in methylcellulose was assessed after washing and incubation for 7 days. (**F**) Downregulation of beclin-1 or ATG7 using siRNA (si) impaired the resensitization of Jurkat cells to dexamethasone by obatoclax or rapamycin compared with scrambled (scr) controls. Cell viability was assessed by MTT. Efficiency of downregulation at 48 hours was analyzed by Western analysis. (**G**) Downregulation of beclin-1 and ATG7 in Jurkat cells protected cells from obatoclax- and dexamethasone-induced cell death in the clonogenic assay. ***$P < 0.05$.

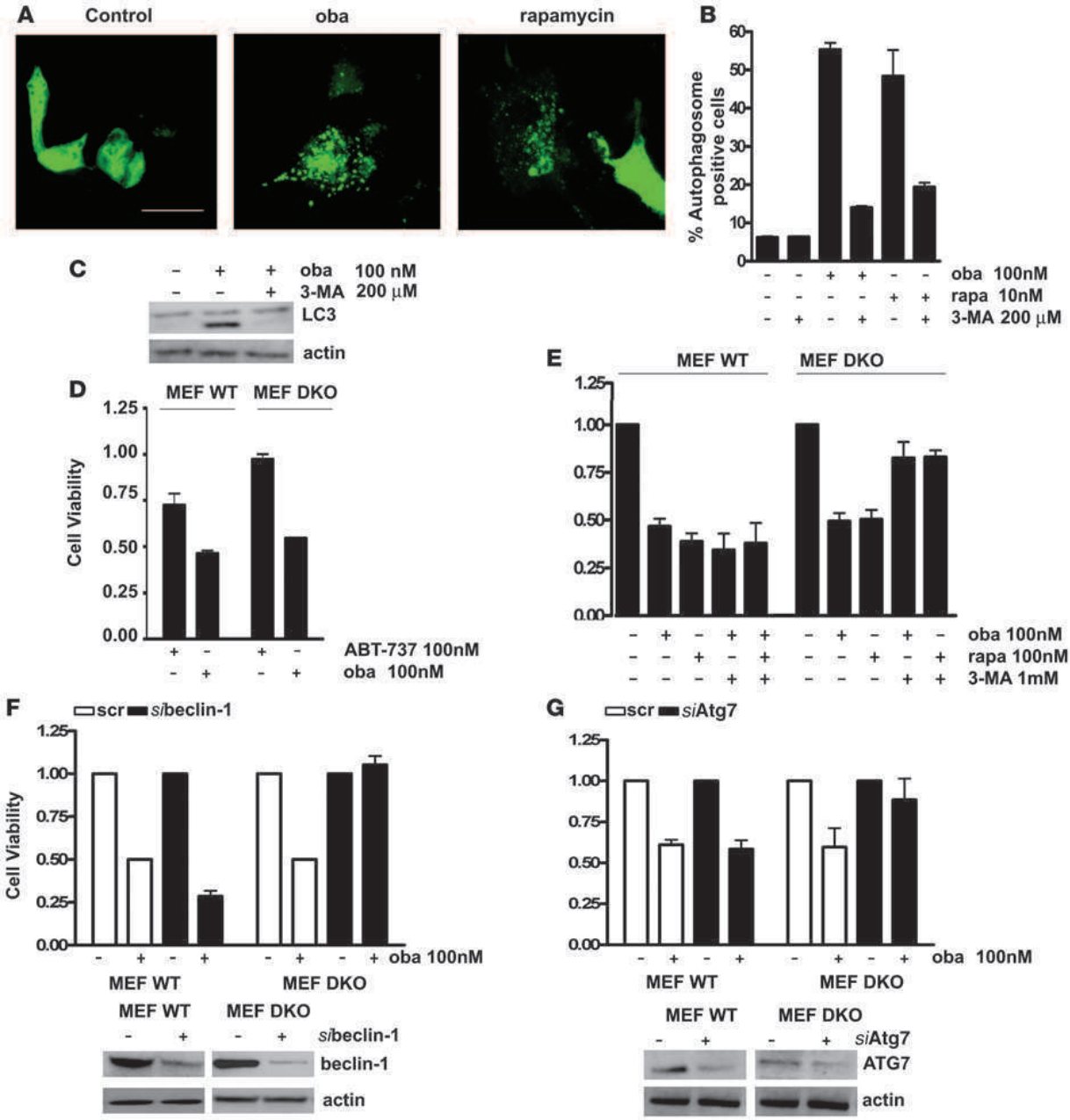


Figure 4

Obatoclax and rapamycin induce autophagic cell death in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. (**A**) *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs transiently expressing the autophagy marker GFP-LC3 were treated with vehicle, obatoclax (100 nM), or rapamycin (10 nM) for 4 hours. Autophagosome formation was monitored by confocal microscopy. Scale bar: 20 μm. (**B**) Quantitation of autophagosome-positive cells. The data represent mean ± SD of 2 independent experiments, counting 200 cells each. (**C**) Treatment of *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs with obatoclax induced generation of endogenous LC3-II as detected by Western analysis, an effect which was blocked by 3-MA treatment. (**D**) WT or *Bax*$^{-/-}$*Bak*$^{-/-}$ (DKO) MEFs were incubated for 48 hours with obatoclax or ABT-737, and cell viability assessed by the MTT assay. (**E**) Inhibition of autophagy by 3-MA rescued *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs but not WT MEFs from cell death induced by 48 hour exposure to obatoclax or rapamycin. (**F**) Downregulation of beclin-1 rescued *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs but not WT MEFs from cell death induced by obatoclax as evaluated by the MTT assay. Efficiency of downregulation was determined by Western blot analysis. (**G**) Downregulation of ATG7 rendered *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs but not WT MEFs resistant to obatoclax treatment. Efficiency of downregulation was assessed by Western blotting.

of dexamethasone in GC-sensitive cells (Figure 2D). In line with these findings, caspase-9 was not activated and caspase-3 was only marginally activated in steroid-resistant cells treated with the combination of obatoclax and dexamethasone (Supplemental Figure 4). Collectively, our results indicate that the combination of obatoclax and dexamethasone triggers a cell death mechanism that is independent of the apoptotic function of caspases in steroid-resistant leukemia.

Induction of autophagy is essential for steroid sensitization in ALL cells. Because an autophagy-dependent cell death pathway has been

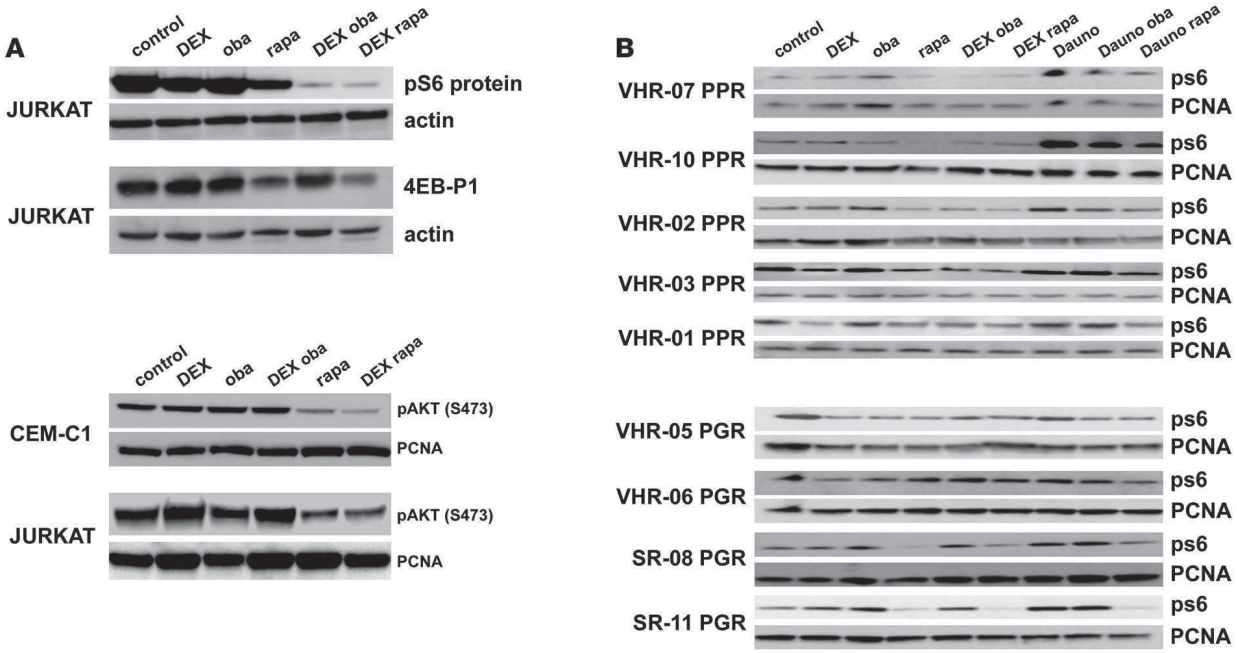


Figure 5

Combination treatment with dexamethasone and obatoclax leads to inhibition of mTOR. (**A**) Jurkat cells were treated for 6 hours as indicated, and phosphorylation of the mTOR targets, S6 protein and 4EB-P1, was assessed. Combination of dexamethasone with either obatoclax or rapamycin resulted in inhibition of mTOR activity (top panel). Rapamycin alone and in combination with dexamethasone, but not obatoclax alone or with dexamethasone, decreased the phosphorylation of AKT at Ser473 in Jurkat and CEM-C1 cells (bottom panel). (**B**) In primary cells from PPR patients with VHR-ALL, treatment with dexamethasone and obatoclax or rapamycin resulted in a decrease of S6 protein phosphorylation. In contrast, in cells from prednisone-good-responder patients, S6 protein was not dephosphorylated after combination treatment.

described in cell line models in which apoptosis is prevented (11, 24), we evaluated next whether autophagy was required for GC sensitization by obatoclax. Indeed, autophagosome formation was evident in GC-resistant cells after 4 hours of exposure to low-dose obatoclax in combination with dexamethasone, which correlated with generation of endogenous LC3-II (Figure 3, A–C). Because rapamycin is known to be an inducer of autophagy (25), we asked whether the GC-sensitizing effect reported recently for rapamycin (5) could also be dependent on autophagy. As we expected, steroid sensitization by rapamycin was associated with hyperautophagic features (Figure 3, A and B). Obatoclax or rapamycin alone only marginally induced autophagosome formation and generation of LC3-II (Figure 3, A–C). Preincubation with the autophagy inhibitor 3-methyladenine (3-MA) abolished autophagosome formation, generation of LC3-II, and GC resensitization by obatoclax and rapamycin (Figure 3, B–D). Both bafilomycin, a late-stage inhibitor of autophagolysosome formation (25), and downregulation of the essential autophagy genes beclin-1 (*BECN1*) and ATG-7 (*ATG7*) by RNA interference blocked GC-resensitization by obatoclax and rapamycin (Figure 3, D–G). These results were confirmed in an independent cell line (Supplemental Figure 5). Clonogenic assays demonstrate that both addition of 3-MA and downregulation of beclin-1 prevented the cytotoxic effect of low-dose obatoclax combined with dexamethasone, since clonogenic growth was comparable to that in untreated controls (Figure 3, F and G). In GC-sensitive ALL cells, inhibition of autophagy by 3-MA only marginally restored GC resistance, despite small amounts of detectable LC3 lipidation (Supplemental Figure 6). These findings implicate autophagy as part of a common mechanism for GC resensitization by obatoclax and rapamycin.

To determine whether obatoclax triggers the autophagy pathway as a general response to blockade of mitochondrial apoptosis, we studied the drug's effects on *Bax*$^{-/-}$*Bak*$^{-/-}$ mouse embryo fibroblasts (MEFs), which exhibit resistance to a broad range of apoptotic stimuli (26). Like rapamycin, obatoclax induced autophagy in *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs (Figure 4, A–C), as reflected by autophagosome formation (Figure 4, A and B) and generation of LC3-II (Figure 4C). Obatoclax was cytotoxic to WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, whereas ABT-737's activity was restricted to WT MEFs (Figure 4D). The death response was blocked by 3-MA (Figure 4E) and downregulation of beclin-1 or ATG-7 (Figure 4, F and G). Taken together, these results indicate that the cytotoxic effects of obatoclax and rapamycin derive from induction of autophagy when mitochondrial apoptosis is blocked.

Modulation of the GC response by obatoclax is associated with inhibition of mTOR. A key regulator of cell fate decisions, including regulation of autophagy, is the kinase mTOR (27). Inhibition of mTOR by rapamycin resensitizes steroid-resistant ALL cells to dexamethasone (5), but the role of autophagy has not been investigated in this study. Since dexamethasone has been shown to inhibit mTOR in other cellular systems (28), we investigated the effect of dexamethasone and obatoclax treatment on mTOR activity. Alone dexamethasone slightly induced a decrease of S6 protein phosphorylation (Figure 5A). Treatment with obatoclax or rapamycin and dexamethasone resulted in a sharp decrease of the phosphorylation of the mTOR targets S6 protein and 4EB-P1 (Figure 5A). Interestingly, rapamycin but not obatoclax, either alone or in combination with dexamethasone, induced dephosphorylation of AKT at Ser473 (Figure 5A). Combination of obatoclax or rapamycin and dexamethasone also resulted in

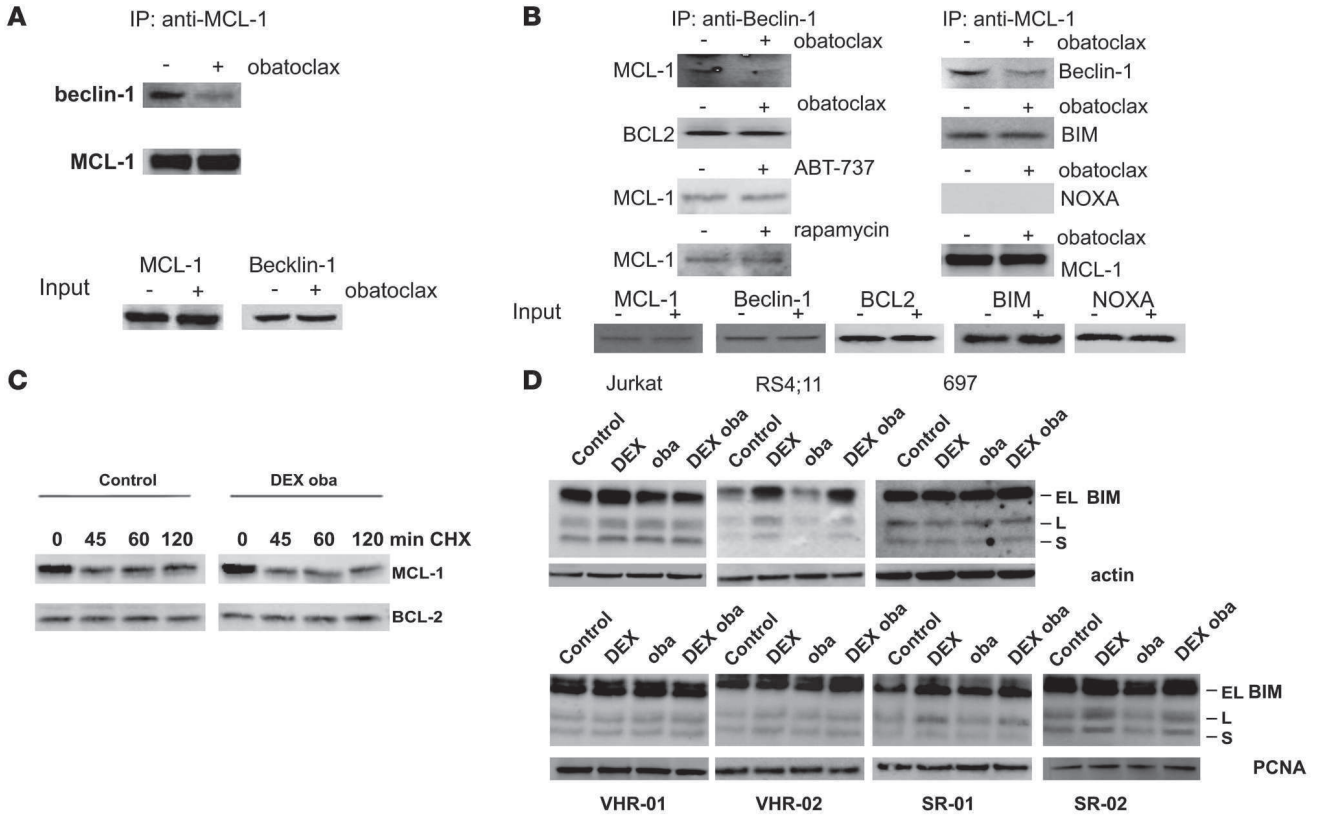


Figure 6

Obatoclax induces disruption of a complex of MCL-1 and beclin-1. (**A**) FLAG-tagged beclin-1 was overexpressed in 293T cells, and MCL-1 immunoprecipitates were analyzed for the presence of beclin-1, with or without obatoclax treatment for 3 hours. (**B**) Jurkat cells were treated with 100 nM obatoclax, 30 nM ABT-737, or 10 nM rapamycin for 3 hours, and MCL-1 or beclin-1 immunoprecipitates were analyzed for the presence of proteins as indicated. Low-dose obatoclax disrupted the interaction between beclin-1 and MCL-1, while interactions between BCL2 and beclin-1 were unaffected. Likewise, ABT-737 or rapamycin did not affect complexes between beclin-1 and MCL-1 or BCL2. (**C**) MCL-1 levels were assessed after treatment with vehicle or the combination of dexamethasone and obatoclax at indicated time points, with or without cycloheximide (CHX). Western blot analyses revealed that MCL-1 levels were unaffected by combination treatment. (**D**) BIM induction was assessed by Western blot in steroid-resistant (Jurkat, VHR-01, and VHR-02) and steroid-sensitive (RS4;11, 697, SR-01, and SR-02) cell lines and primary cells treated with DEX (1 μM), obatoclax (100 nM), or the combination for 24 hours.

dephosphorylation of S6 protein in primary ALL cells from steroid-resistant high risk ALL patients (Figure 5B, left). In contrast, cells from steroid-sensitive patients did not show dephosphorylation of S6 protein after treatment with obatoclax and dexamethasone (Figure 5B, right). Our experiments suggest that in the context of GC resistance, exposure to dexamethasone could cooperate with exposure to obatoclax to decrease mTOR activity. This finding will be exploited to develop correlative markers of biological response to in vivo treatment, for example, using phospho-flow cytometry to monitor the phosphorylation status of S6 protein in leukemia cells.

Obatoclax disrupts a complex between beclin-1 and MCL-1. The antiapoptotic BCL-2 family member MCL-1 was proposed to act as a modulator of GC resistance (5). Because the autophagy regulator beclin-1 was described to interact with antiapoptotic BCL-2 family members, including MCL-1 (11, 14, 15), we next tested the effect of obatoclax on the interaction of beclin-1 with MCL-1. Subcytotoxic concentrations of obatoclax resulted in a markedly decreased detection of MCL-1 that coimmunoprecipitated with overexpressed, epitope-tagged beclin-1 in 293T cells (Figure 6A). We confirmed this result by performing coimmunoprecipitation experiments of endogenously expressed proteins in ALL cells (Figure 6B). Again, in presence of subcytotoxic concentrations of obatoclax, less beclin-1 was shown to coimmunoprecipitate with MCL-1, and inversely, less MCL-1 was pulled down together with beclin-1. Obatoclax did not modify interactions between beclin-1 and BCL-2. Interestingly, incubation with rapamycin or ABT-737 did not affect the complex between beclin-1 and MCL-1. As expected from the reported MCL-1 interaction spectrum (29, 30), BIM was detectable by coimmunoprecipitation from steroid-resistant ALL cells, but binding of BIM to MCL-1 was not altered by treatment with low-dose obatoclax. The BH3-only protein NOXA, which was shown to interact preferentially with MCL-1, did not bind to MCL-1 in this experimental setting (Figure 6B).

Since MCL-1 can be subjected to a high protein turnover (31), we evaluated the effect of combination treatment with obatoclax and dexamethasone on MCL-1 protein levels. In the presence of cycloheximide, MCL-1 levels rapidly decreased after 45 minutes of incubation, independent of the presence of obatoclax and dexamethasone (Figure 6C). Absence of proapoptotic BIM induction by dexamethasone has been described in selected cases of steroid-resistant ALL (32). We did not detect any changes in BIM expression levels to correlate with the restored response to dexamethasone in combination with obatoclax

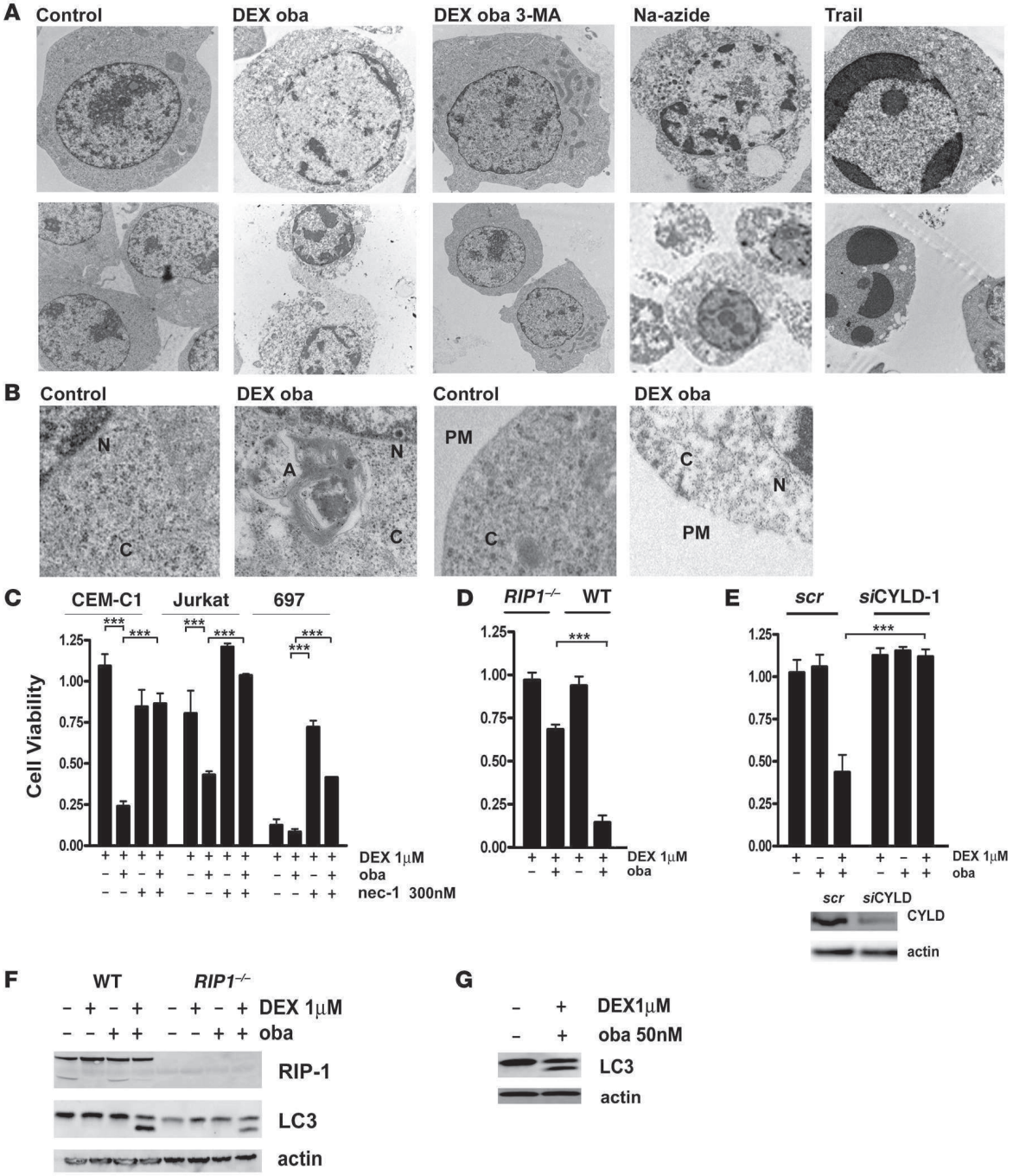
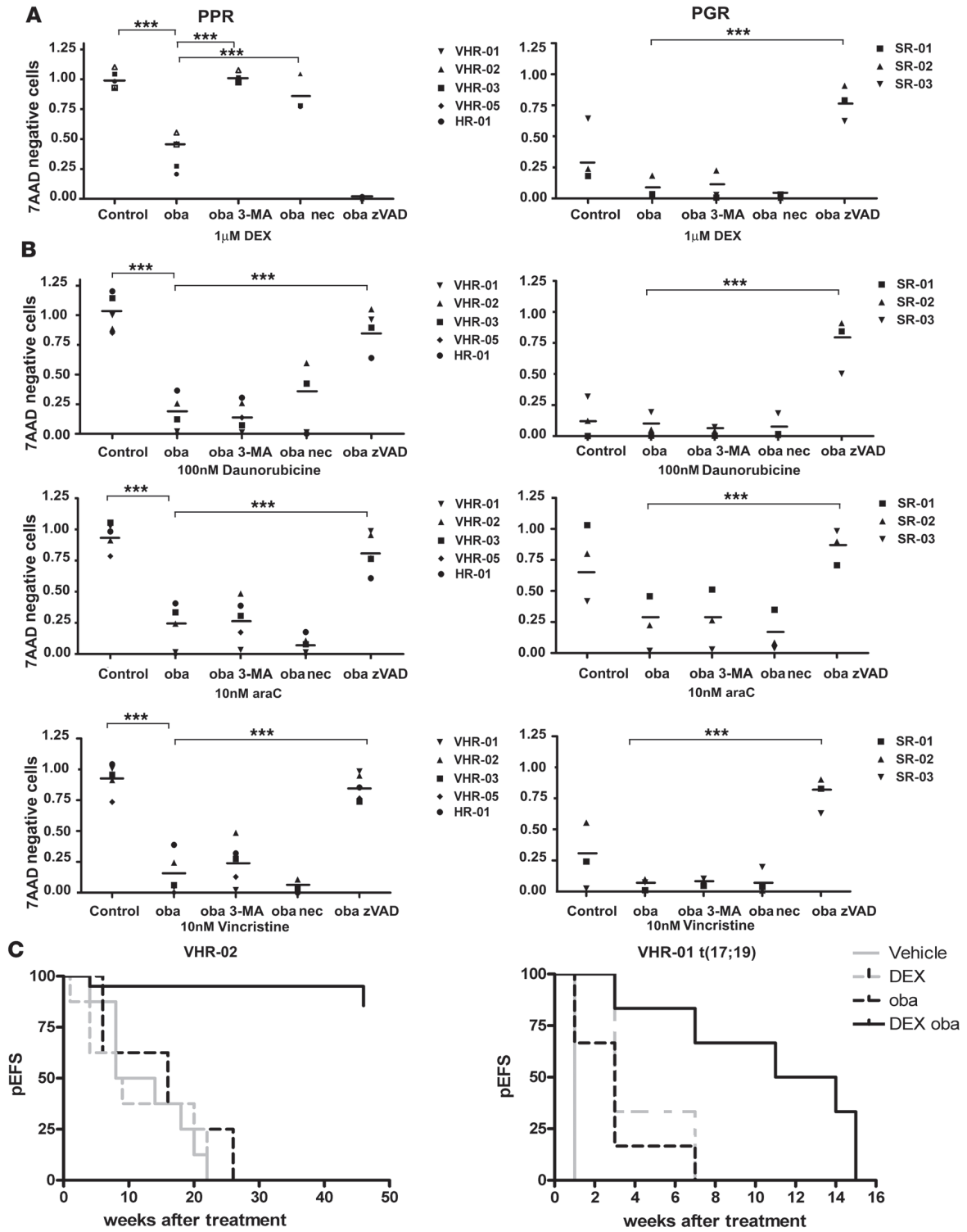


Figure 7

RIP-1 kinase activity is essential for obatoclax-mediated GC sensitization. (**A**) Electron microscopy images reveal features of necroptotic cell death after treatment for 72 hours with obatoclax and dexamethasone. No condensed chromatin was detectable, while Trail treatment induces condensed apoptotic nuclei (top panel) (original magnification, ×7,100). Cells treated with obatoclax and dexamethasone exhibited disintegrated plasma membranes, which was recapitulated by Na-azide. Trail treatment leaved membranes intact (bottom panel) (original magnification, ×5,400). (**B**) A more detailed view of the same experiment. An autophagosome formation with characteristic double membrane structures was detected in the cytoplasm. The plasma membrane was disrupted in cells treated with obatoclax and dexamethasone. N, nucleus; C, cytoplasm; A, autophagosome; PM, plasma membrane (original magnification, ×15,000). (**C**) In steroid-resistant (CEM-C1 and Jurkat) and steroid-sensitive (697) cells treated for 72 hours with dexamethasone (1 μM) and obatoclax (10% IC_{50}), with or without the necroptosis inhibitor nec-1 (300 nM), nec-1 restored steroid resistance as assessed by the MTT assay. (**D**) Jurkat *RIP1*$^{-/-}$ cells were less sensitive to the double treatment for 72 hours with dexamethasone (1 mM) and obatoclax (10% IC_{50}) compared with WT Jurkat cells. Cell viability was assessed by the MTT assay. (**E**) Downregulation of CYLD rescued Jurkat cells from cell death induced by combination treatment for 72 hours with dexamethasone (1 μM) and obatoclax (10% IC_{50}). Efficiency of downregulation was assessed by Western blot analysis after 48 hours. (**F**) LC3-II generation occurred in Jurkat *RIP1*$^{-/-}$ cells and in WT cells after 8 hours of treatment with dexamethasone and obatoclax. (**G**) Treatment with nec-1 did not inhibit LC3-II generation induced by treatment with obatoclax and dexamethasone in Jurkat cells. ***$P < 0.05$.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Control
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Vehicle
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Figure 8
Obatoclax displays a strong chemosensitizing activity in multidrug-resistant primary ALL cells from poor risk patients. (**A**) Primary ALL cells from 5 PPR patients and 3 prednisone-good-responder patients were cocultured with hTERT-immortalized bone marrow stroma cells and treated with either dexamethasone (1 μM) alone or in combination with obatoclax (10% IC_{50}) in the presence or absence of 3-MA, nec-1, or zVAD.fmk for 72 hours. Cell viability was assessed by 7AAD staining and flow cytometry. Data are shown as mean ± SD of 2 independent experiments. In combination experiments, values were normalized to cells treated with compounds and/or inhibitors alone at indicated dose. (**B**) Primary ALL cells from 4 VHR, 1 high risk (HR), and 3 standard risk (SR) patients were cocultured with hTERT-immortalized bone marrow stroma cells and treated with either daunorubicin, vincristine, or cytarabine (araC) alone or in combination with obatoclax (10% IC_{50}) in the presence or absence of 3-MA, nec-1, or zVAD.fmk for 72 hours. Cell viability was assessed by 7AAD staining and flow cytometry. Data are shown as mean ± SD of 2 independent experiments. ***$P < 0.05$ (**A** and **B**). (**C**) Percentage of event-free survival (pEFS) of NSG mice after xenotransplantation with primary cells from 2 PPR patients with VHR-ALL (VHR-02, $n = 8$; patient VHR-01, $n = 6$; Supplemental Table 2) and treatment for 3 weeks with either vehicle, dexamethasone, obatoclax, or the combination. $P < 0.02$ for dexamethasone and obatoclax versus vehicle, dexamethasone, or obatoclax alone for both xenograft experiments.

in GC-resistant ALL cells (Figure 6D). Interestingly, induction of BIM was not detectable consistently in steroid-sensitive cells (Figure 6D). While in RS4;11 cells, BIM was readily induced by dexamethasone, neither 697 cells nor primary ALL cell samples from steroid-resistant patients showed induction of this proapoptotic BH3-only protein after dexamethasone treatment (Figure 6D). In cells from steroid sensitive patients, a small increase in the BIM-L and BIM-S but not BIM-EL isoforms was detectable. These data indicate that steroid resensitization by obatoclax is not due to induction of BIM.

To obtain further insight into the role of MCL-1 for the mechanism of action of obatoclax, we modulated MCL-1 expression using RNA interference. A reduction of MCL-1 protein levels resulted in partial resensitization to dexamethasone but, surprisingly, prevented any additional chemosensitization by the combination with obatoclax (Supplemental Results and Supplemental Figure 7). Partial resensitization to dexamethasone was mediated via induction of apoptosis, which was blocked using the pan-caspase inhibitor zVAD.fmk. Inhibition of autophagy with 3-MA did not affect the resensitization effect of MCL-1 knockdown to dexamethasone, even in presence of obatoclax. Instead, caspase-9 activation was detected, which correlated with a caspase-dependent decrease of beclin-1. Thus, it is possible that induction of apoptosis overrides induction of autophagy when MCL-1 is downregulated, which limits the interpretation of these experiments. Taken together, our data indicate that disruption of the beclin–MCL-1 interaction is part of the mechanism triggered by obatoclax and suggest an involvement of MCL-1 in the regulation of an autophagy-dependent cell death pathway, in addition to its role as anti-apoptotic regulator in leukemia cells.

Combination of obatoclax and dexamethasone triggers RIP-1 kinase–dependent necroptosis in GC-resistant ALL. To better assess the nature of cell death occurring with obatoclax and dexamethasone, we proceeded with imaging by transmission electron microscopy. Indeed, autophagosome formation was evident after treatment with obatoclax and dexamethasone, which was completely inhibited by incubation with 3-MA (Figure 7, A and B). Cells displayed features consistent with a nonapoptotic cell death mechanism after exposure to obatoclax and dexamethasone. Condensation of chromatin, a hallmark of classical apoptosis, was incomplete (Figure 7A, top row), which contrasts with the marked chromatin condensation after treatment with TRAIL, which induces death receptor–dependent apoptosis in ALL cells (33). A characteristic feature of dead cells was the disintegration of the plasma membrane (Figure 7B), which is a hallmark of necroptosis (34). Increasing evidence suggests that a programmed necrotic cell death pathway can be triggered in the context of impaired apoptotic effectors, and this is dependent on RIP-1 kinase activity (12, 17). RIP-1 functions at the intersection of cell survival and cell death signalling (35, 36). Therefore, we tested whether inhibition of RIP-1 with necrostatin-1 (nec-1; ref. 17) would interfere with steroid sensitization by obatoclax. Indeed, steroid-resistant ALL cells treated with nec-1 were refractory to obatoclax-induced dexamethasone resensitization (Figure 7C). Nec-1 also reduced the cytotoxic response to dexamethasone, alone or in combination with low-dose obatoclax in steroid-sensitive ALL cells (Figure 7C). Interestingly, nec-1 inhibited induction of BIM in steroid-sensitive cells that showed BIM induction after dexamethasone (Supplemental Figure 8). Consistent with a central role for RIP-1 for the response to GC drugs, RIP-1–deficient ALL cells (37) were resistant to obatoclax-mediated GC resensitization (Figure 7D).

The deubiquitinase cylindromatosis (turban tumor syndrome) (CYLD) is a direct regulator of RIP-1 kinase activity (38). Knocking down CYLD expression showed that CYLD is required for RIP-1–dependent cell death induction by combination of dexamethasone and obatoclax (Figure 7E). Our results are consistent with the current model, which proposes that the deubiquinated form of RIP-1 is mediating cell death signals (35). An important finding is that induction of autophagy could be detected early, within 4 hours of treatment, and that generation of LC3-II was neither inhibited in RIP-1–deficient ALL cells (Figure 7F) nor in nec-1–treated ALL cells (Figure 7G). Taken together, these results demonstrate that the combination of obatoclax and dexamethasone triggers a non-apoptotic cell death pathway, which shares the central features of necroptosis, including the characteristic morphology of cell death shown by electron microscopy, and a strict dependence on RIP-1 kinase activity and RIP-1 deubiquitinase CYLD. Moreover, our data indicate that the autophagic process is triggered upstream of RIP-1 and does not occur as a consequence of necroptosis.

Obatoclax is a broad chemosensitizer in multidrug-resistant primary ALL cells, but activation of autophagy-dependent cell death is specific to the combination with dexamethasone. To validate our results in primary cells, we tested pretreatment samples from PPR patients with precursor B cell ALL, who were further characterized by the molecular persistence of leukemic cells under intensified conventional multiagent chemotherapeutic treatment and predicted to have a very high risk of relapse based on their poor in vivo response to chemotherapy (Supplemental Table 2). Indeed, low-dose obatoclax resensitized GC-resistant ALL cells to dexamethasone in mesenchymal stroma cell (MSC) cocultures, which was completely abolished using 3-MA, implicating autophagy as an essential mechanism in primary refractory ALL cells also (Figure 8A). At this dosage, obatoclax alone induces less than 5% cell death. Importantly, 3-MA did not interfere with the cytotoxic effect of treatment with dexamethasone, alone or combined with obatoclax, on primary ALL cells from GC-sensitive patients (Figure 8A), indicating that autophagy was not required for the death response to GC drugs in GC-sensitive cells. Consistent with our results in ALL cell lines, nec-1 abolished the GC-sensitizing activity of combination treatment in steroid-resistant primary

VHR-ALL cells. In contrast, blocking caspases with zVAD.fmk did not inhibit steroid resensitization in primary GC-resistant ALL cells, while the response to dexamethasone was inhibited by zVAD.fmk treatment in cells from steroid-sensitive ALL patients.

Current treatment protocols include a steroid window followed by multidrug chemotherapy, including cytotoxic agents such as daunorubicin, vincristine, and cytarabine. Low-dose obatoclax resensitized primary refractory ALL cells to all 3 drugs, while the sensitivity of cells from sensitive patients was not increased further. Interestingly, 3-MA did not restore resistance to these cytotoxic agents, indicating that induction of autophagy after treatment with obatoclax is specific for GC resensitization (Figure 8B). In contrast, blocking caspases using zVAD.fmk completely inhibited the resensitizing effect of low-dose obatoclax in combinations with daunorubicin, vincristine, and cytarabine, both in cells from very high risk and from standard risk patients. These results support the notion that autophagy-dependent necroptosis is specifically triggered with GCs in a GC-resistant context. Moreover, obatoclax could serve as a chemosensitizer for established multidrug regimens for ALL treatment, given its broad potential to restore the apoptotic response with non-GC antileukemic drugs.

Combination of obatoclax and dexamethasone is effective in a leukemia xenograft model, using cells from refractory ALL patients. To test the in vivo efficacy of obatoclax in GC resensitization, we transplanted primary ALL cells from 2 VHR-PPR patients into NOD/SCID/IL2rγ^{null} (NSG) mice (39). When 1% human leukemic cells were detected in mouse peripheral blood, we treated mouse cohorts with vehicle, obatoclax (5 mg/kg/d), dexamethasone (5 μg/kg/d), or both for 3 weeks. In vehicle-treated animals, progression to leukemia (>10% peripheral blasts) was observed in a median time of 11 weeks for animals xenografted with cells from patient VHR-02 and 1 week for animals xenografted with cells from patient VHR-01. All mice xenografted with cells from patient VHR-02 and treated with single-agent dexamethasone or obatoclax had to be sacrificed due to leukemia progression, while only 2 out of 8 animals treated with the combination of obatoclax and dexamethasone progressed to leukemia over the 50-week observation period. A significant delay in leukemia progression was achieved by combination treatment with dexamethasone and obatoclax when cells from VHR-01 were used ($P < 0.02$ for combination treatment versus vehicle or single agent treatment). This patient showed the recurrent translocation t(17;19)(q22;p13), which is virtually always associated with early relapse (40) (Figure 8C). This result suggests that GC sensitization of VHR-PPR ALL cells can be achieved in vivo, providing a strong rationale to explore the therapeutic potential of combined dexamethasone and obatoclax clinically.

Discussion

Here we describe a pharmacological approach that we believe to be new to specifically bypass the apoptotic blockade to chemotherapy in multidrug-resistant ALL. Subcytotoxic concentrations of the BCL-2 antagonist obatoclax restored the response to dexamethasone by inducing a nonapoptotic cell death pathway. The activation of autophagy-dependent cell death in cells that are resistant to the apoptotic stimuli by dexamethasone is reminiscent of observations that were reported using experimental systems with defined genetic defects of key regulators of the intrinsic and extrinsic apoptotic response. In *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs and BCL-2–overexpressing MEFs, cell death triggered by etoposide or STS was dependent on autophagy genes beclin-1 and ATG-5 (11). As also reported by others (6, 8), obatoclax was cytotoxic for *Bax*$^{-/-}$*Bak*$^{-/-}$ cell lines, which we demonstrate to be critically dependent on the autophagy pathway. Similarly, genetic or pharmacologic interference with caspase 8 or death receptor signalling can result in autophagy-dependent cell death with necroptotic features (12, 18, 41). In lymphoid cells, this may constitute an alternative mechanism to control abnormal cellular proliferation in the absence of a normal apoptotic response. In activated T cells from mice with caspase-8 or FADD deficiency, autophagic signalling was required to induce RIP-1–dependent necroptotic cell death (41). We here show that this mechanism of cell death can be activated in GC-resistant leukemia to restore the response to dexamethasone.

Autophagy has been recognized as an important regulatory mechanism of cell fate decisions. While it is clear that autophagy can have a protective function at times of cellular stress, the contribution of autophagy to the execution of programmed cell death is a subject of controversy (13). Our data demonstrate that autophagic signalling is an integral part of the cell death mechanism when the response to dexamethasone is restored with obatoclax or rapamycin. Both inhibitors that interfere early (3-MA) or late (bafilomycin) with the autophagic process and knockdown of genes that are essential for autophagy, *BECN1* and *ATG7*, prevented resensitization to dexamethasone completely. Combination of dexamethasone and obatoclax inhibited clonogenic growth of GC-resistant ALL cells, and electron microscopy imaging unequivocally showed rapid induction of necrotic cell death with autophagic features. There is clear evidence that in cancer, autophagy is not necessarily a protective feature. For example, *BECN1* was shown to act as a haploinsufficient tumor suppressor gene (42, 43), with increased frequency of spontaneous neoplasia, including lymphomas in beclin-1–haploinsufficient mice. The genes that are essential for the autophagic machinery are highly conserved and required in several autophagy-dependent cellular processes (44). Several studies link autophagy genes to programmed cell death. In the central nervous system for example, ATG7 deficiency protected neurons from caspase-dependent and caspase-independent cell death after hypoxic/ischemic brain injury (45). In human glioblastoma, knockdown of the autophagy genes *ATG1* or *ATG5* prevented the cytotoxic effect of cannabinoids, which induce autophagy-dependent cell death via an mTORC1-dependent pathway (46). Autophagy was also shown to regulate programmed cell death during development. The steroid hormone ecdysone triggered autophagy-dependent cell death during morphogenesis of salivary glands from the larval stage to the adult stage in *Drosophila*. This cell death pathway was independent of caspase activity (47), providing compelling evidence for the modulation of an autophagic cell death pathway via steroid hormone signalling in normal development. Similarly, autophagy is required for programmed cell death in the midgut during *Drosophila* metamorphosis, which provides additional evidence for specific regulation of cell death by autophagy, even in presence of an intact apoptotic machinery (48). Combination treatment of obatoclax with GC drugs but not with other cytotoxic agents induced autophagy-dependent cell death in resistant ALL. This raises the question of whether autophagy is also required for the effect of dexamethasone in GC-sensitive ALL. A recent report described increased autophagy after dexamethasone treatment in GC-sensitive ALL cell lines, but cell death was associated with apoptotic features and knockdown of beclin-1 resulted only in partial rescue of this effect (49). We also observed a partial reduction of dexamethasone cytotoxicity using 3-MA (Supplemen-

tal Figure 6) or nec-1 (Figure 7C) in a subset of GC-sensitive cell lines. We could, however, not detect an effect of 3-MA in primary ALL cells, in which caspase-dependent cell death prevailed. Our results identify autophagy as an early and limiting step to steroid sensitization by obatoclax in GC-resistant cells and underscore the importance of understanding the cellular context when designing strategies to target autophagy for cancer treatment.

There is clear evidence for hyperactivation of AKT (50) and mTOR (5) in GC-resistant ALL. Because mTOR is implicated in the control of autophagy in different settings (27), hyperactive AKT-mTOR signalling could prevent induction of autophagy in resistant disease. We hypothesized that GC-resistant ALL cells could therefore be primed for mTOR-controled autophagy. Consistent with this idea, we found that induction of autophagic cell death by the combination of dexamethasone and obatoclax resulted in marked reduction in phosphorylation of the mTOR target S6 protein in resistant cells. The mechanisms by which obatoclax or rapamycin potentiate the effect of dexamethasone on mTOR appear to be different. Indeed combination of dexamethasone with rapamycin, but not with obatoclax, resulted in a marked decrease in phosphorylation of AKT on Ser473, consistent with recent finding showing that mTOR can also act upstream of AKT (51). Modulation of mTOR target phosphorylation was only seen when obatoclax was combined with dexamethasone, but not with other cytotoxic agents, suggesting that dexamethasone exposure contributes to inhibition of mTOR. Indeed, exposure to dexamethasone was reported to be associated with repression of mTOR signalling in myoblast cell lines and lymphoid cells (28, 52). The importance of mTOR for the control of autophagy is also underscored by the results of a comprehensive screen using a chemical compound library in order to identify new pharmacologic inducers of autophagy, in which proautophagic activity of candidate molecules was always associated with decreased phosphorylation of mTOR targets (53). Extensive studies will be required to dissect the primary signalling events triggered by the combination of obatoclax and dexamethasone, as they may provide important clues about the mechanisms of drug resistance in ALL.

GC resistance does not appear to be associated with genetic or functional defects of the GC receptor in ALL (54, 55). Dexamethasone was proposed to induce apoptosis by increasing the levels of the BH3-only proapoptotic protein BIM, which were markedly reduced in selected cases of GC-resistant ALL (32). However, in most cases tested, we could not detect induction of BIM by dexamethasone in GC-sensitive cell lines and primary ALL cells. Furthermore, we did not detect increased levels of BIM, decreased MCL-1 protein levels, or increased MCL-1 protein turnover associated with steroid sensitization. Instead, we found, by immunoprecipitation of endogenous proteins, subcytoxic concentrations of obatoclax to result in the disruption of MCL-1 with beclin-1 in ALL cells. This suggests the possibility that MCL-1 could control induction of autophagy via beclin-1, as it was proposed for other BCL-2 family members recently (14, 15). Interestingly, this effect was not seen in cells treated with rapamycin, again pointing out that the target mechanism of obatoclax is different. We recognize the fact that this effect could also result from indirect mechanisms on the protein complex, including MCL-1 and beclin-1. Furthermore, functional experiments modulating MCL-1 expression levels were not conclusive. Knockdown of MCL-1 resulted in moderate activation of apoptosis and partial sensitization to dexamethasone that was caspase dependent but prevented complete resensitization to dexamethasone by obatoclax and autophagic cell death. Activation of apoptosis is possibly overriding induction of autophagy in this context. In support of this hypothesis, we detected activation of caspase-9 after downregulation of MCL-1, with caspase-9–dependent cleavage of beclin-1. This provides a possible mechanism to prevent autophagy induction when apoptosis is activated. Taken together, steroid modulation with low-dose obatoclax did not involve release of proapoptotic BCL-2 family proteins from MCL-1 but triggered autophagy-dependent cell death by a mechanism that required the presence of MCL-1.

In this context, activation of autophagy is required to induce necroptosis, a form of programmed necrosis that has been described to occur when apoptosis is abortive due to caspase inhibition (16, 56). Execution of necroptosis is dependent on the RIP-1 kinase (17). Our data demonstrate that RIP-1 activity is absolutely required for steroid sensitization by obatoclax and by rapamycin. The cell death morphology that was documented by electron microscopy is consistent with the morphology reported for necroptosis (12, 56). Furthermore, the deubiquitinase cylindromatosis (CYLD), which has been shown to regulate RIP-1 (38), was functionally required for execution of necroptosis. This is consistent with experiments showing that RIP-1 kinase activity was required to trigger cell death and that ubiquitination of RIP-1 prevents cell death signalling (16, 19). RIP-1 and CYLD were included among the core genes that were identified in a functional siRNA screen for genes that were essential for necroptosis (18). To the best of our knowledge, we provide the first clinically relevant model with consistent validation in primary leukemia cells from patients with very high risk disease, in which the necroptotic pathway can be exploited to restore response to therapy. As such, this will constitute a very relevant experimental model to study the mechanisms of necroptosis in depth. Our results clearly imply a direct link between the autophagic pathway and RIP-1–mediated signalling events leading to necroptosis. Autophagy is triggered early and independent of RIP-1 kinase activity, indicating that it acts upstream of necroptotic signals. A number of studies identify RIP functionally as part of a complex with proteins of the death receptor pathway, such as FADD and caspase-8 (35). Experiments using mouse models indicate that autophagy and necroptosis could be linked via recruitment of components of the death receptor pathway to the membrane of autophagosomes (41). It is tempting to speculate that a similar mechanism is triggered in ALL cells upon costimulation with dexamethasone and obatoclax. Our findings warrant extensive biochemical follow-up studies to understand how RIP-1 is activated and how the autophagic machinery is connected to the necroptotic pathway.

Based on promising studies by others (32), we have established a leukemia xenograft model of de novo highly resistant ALL. The power of this approach resides in the possibility to select cases from relevant patient groups, starting from cryopreserved leftover diagnostic samples, from one of the largest cooperative trials for the treatment of childhood ALL. By focusing on cases with VHR-ALL by MRD, we also selected for patients that are most significantly resistant to prednisone in vivo, as defined by the reduction of leukemia cells in the peripheral blood after 1 week of prednisone monotherapy. Accordingly, ALL cells from these patients were completely resistant to dexamethasone and other chemotherapeutic agents in vitro. Low-dose obatoclax restored the response to dexamethasone, both in precursor B cell and T cell ALL cases. The durable remissions observed with 1 year follow-up,

using the leukemia xenograft model, are indicative of strong antileukemic activity. Furthermore, the broad chemosensitizing effect of low-dose obatoclax in combination with daunorubicin, vincristine, and cytarabine in multidrug-resistant primary ALL cells provides a strong basis for further evaluation of obatoclax in combination with a multidrug regimen. The xenograft approach will be essential to validate this approach for heavily pretreated relapse and refractory patients. Our observation that isolated clones can emerge in clonogenic assays after treatment of a cell line with obatoclax and dexamethasone indicates that resistance to this approach may occur. Our established xenograft system will enable us to screen a larger number of ALL cases to verify whether resistance to this approach has to be expected. The identification of resistant cases would provide a model to establish markers that correlate with response or resistance. Based on current data, dynamic changes of mTOR activity with treatment represent a good candidate marker. Such knowledge will serve to optimize patient selection for clinical trials.

Taken together, our data support a model in which the apoptotic blockade in GC-resistant ALL cells can be overcome by activating an autophagy-dependent necroptotic cell death pathway. The characteristic necroptotic features by electron microscopy and the changes in the phosphorylation profile of S6 protein provide tools to assess the biological response to combination treatment with obatoclax and dexamethasone in patients in refractory ALL. Given the acceptable toxicity profile of obatoclax in clinical studies in adults with hematologic malignancies (9, 10), our study provides a compelling rationale for the evaluation of this new pharmacological strategy for the treatment of children with refractory and relapsed ALL.

Methods

Cell culture, reagents, and standard procedures. CEM-C7-14 and CEM-C1-15 cells (referred to CEM-C7 and CEM-C1 in the text) were provided by E.B. Thompson (University of Texas Medical Branch, Galveston, Texas), human *hTERT*-immortalized primary bone marrow MSCs (57) were provided by D. Campana (St Jude Children's Research Hospital, Memphis, Tennessee), caspase-9–deficient and retransfected Jurkat cells (58) as well as *Bax/Bak*-deficient Jurkat cells (59) were provided by K. Schulze-Osthoff (University of Tübingen, Germany), and parental and *Bax/Bak*-deficient MEFs (26) were provided by J.-C. Martinou (University of Geneva, Switzerland). *Rip1*$^{-/-}$ cells were provided by B. Seed (Harvard Medical School, Boston, Massachusetts). Obatoclax was provided by J. Viallet and G. Shore (Gemin X, Malvern, Pennsylvania) and ABT-737 was provided by S. Elmore and S. Rosenberg (Abbott Laboratories, Chicago, Illinois).

Detailed procedures are described in Supplemental Methods. For RNA silencing experiments, 30 nM beclin-1 (sc-29797, Santa Cruz Biotechnology Inc.) was used. pEGFP-LC3 was provided by M. Jäättelä (Danish Cancer Society, Copenhagen) and the FLAG-tagged beclin-1 construct by B. Levine (University of Texas Southwestern Medical School, Dallas). All constructs were transfected by nucleofection (Amaxa) using solution V and Amaxa program T-016 for CEM-C1, A-024 for CEM-C7 cells, and U-020 for MEFs. Transfection efficiency was controlled using the pEGFP plasmid (Clontech).

Patient samples. ALL cells were recovered from cryopreserved anonymized samples from patients who were enrolled in the ongoing ALL-BFM 2000 protocol and had given informed consent in accordance with the Declaration of Helsinki. Approval was obtained from the Institutional Review Board (IRB) of the Medical School Hannover and the local IRB for all participating centers in the trial ALL-BFM 2000. This approval extends to the use of leftover diagnostic material for add-on research projects, including those addressing basic biological questions.

In vitro drug response. In vitro drug response curves in cell lines were established with the MTT assay and were normalized to vehicle control. Primary patient samples were cocultured on hTERT-immortalized human MSCs, and drug response curves were analyzed with flow cytometry using propidium iodide.

Xenograft model. Xenograft experiments were approved by the veterinary office of the Canton of Zurich. In brief, primary ALL cells were recovered from cryopreserved samples and transplanted intrafemorally to NSG mice. Leukemia progression was monitored by flow cytometry with human CD45 and CD19 antibodies (AbD Serotec). ALL cells recovered from spleens of NSG mice after the first xenotransplantation were used for in vivo therapeutic trials. Randomized cohorts were treated with vehicle intramuscularly (i.m.): 3 mg/kg/d obatoclax i.m., 5 mg/g/d dexamethasone i.p., or the combination of dexamethasone i.p. and obatoclax i.m. for 5 days per week for 3 consecutive weeks.

Statistics. All experiments were performed 4 times, unless stated otherwise in the figure legends. Data are represented as mean ± SD. For in vitro experiments, Student's *t* test (2-tailed) was used on triplicates. *P* values of less than 0.05 were considered significant. Event-free survival (EFS) was defined as the time from the start of treatment until 10% of human leukemia cells were detectable in the mouse peripheral blood and was assessed by Kaplan-Meier analysis. Survival curves were analyzed with the log-rank test (GraphPad prism). *P* values of less than 0.05 were considered significant.

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Address correspondence to: Jean-Pierre Bourquin, Department of Oncology, University Children's Hospital Zurich, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland. Phone: 41.44.266.7304; Fax: 41.44.266.7171; E-mail: jean-pierre.bourquin@kispi.uzh.ch.

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Supplementary results

We performed cell viability and clonogenic assays to assess the effect of MCL-1 knock-down by RNA interference in GC-resistant ALL cells. Reduction of MCL-1 expression levels resulted in a partial increase in dexamethasone sensitivity of GC-resistant ALL cells. This was reversed by zVAD.fmk but not with 3-MA (Figure S7A), as assessed using 7AAD stainings by flow cytometry (upper panel) and in clonogenic assays (lower panel). Moreover, MCL-1 knock-down suppressed the marked GC-sensitization effect by obatoclax that was observed in cells transfected with scrambled siRNA. In addition, reduction of MCL-1 levels resulted in activation of caspase-9 and in induction of mitochondrial membrane depolarization in cells treated with dexamethasone and obatoclax (Figure S7B and C), confirming that the partial GC-sensitization effect occurs by activation of apoptosis. As reported recently, apoptosis can suppress autophagy by enhancing caspase-mediated cleavage of beclin-1 (27). Indeed, exposure to dexamethasone and obatoclax resulted in a decrease of beclin-1 levels after knockdown of MCL-1 (Figure S7D). The decrease in beclin-1 levels did not occur in cells with intact MCL-1 levels and was prevented by zVAD.fmk or in cells devoid of caspase-9. Taken together, our results show that MCL-1 is involved in preventing apoptosis induction in GC-resistant ALL cells, and that in absence of MCL-1, dexamethasone and obatoclax trigger mitochondrial apoptosis, which overrides the autophagy-dependent pathway.

Supplementary methods

Reagents

Dexamethasone was purchased from Mepha Pharma, rapamycin from Calbiochem, and 3-MA from Sigma. Obatoclax was provided by GEMIN X, ABT-737 by Abbott Laboratories, and rabbit antibodies were from Cell Signaling Technology (anti-Beclin-1, 1:1000 and anti-Bcl-X_L, 1:1000), Sigma (anti-actin, 1:3000 and anti-Mcl-1, 1:3000), ProSci (anti-APG7, 1:1000) and Abgent (anti-APG5L, 1:1000). Mouse-anti-LC3 (1:1000) was purchased from Axxora. FITC-labelled anti-human CD45, PE-labelled anti-human CD19 and Alexa 647-labelled anti-mouse CD45 antibodies were from Serotec.

Cell culture

The ALL cell lines, CEM-C1 and -C7, 697 (ATCC #CRL-7433), MOLT-4 (ATCC #CRL-1582), Jurkat (ATCC #TIB-152) and REH (DSMZ, ACC22) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin (Invitrogen). The RS4;11 (ATCC # CRL-1873) cell line was maintained in alpha MEM medium containing 10% heat-inactivated fetal bovine serum and 100 IU/mL penicillin/streptomycin (Invitrogen). Human mesenchymal stroma cells (MSC) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 50µM hydrocortisol (Sigma). Wild-type primary mouse embryonic fibroblasts and those deficient for BAX and BAK were maintained in DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin. For all compounds, the effect of treatment at low dose was used to normalize values for the calculation of survival curves.

Immunoprecipitations and western analysis

For immunoprecipitation experiments, treated cells were lysed in CHAPS-buffer (20 mM Tris-Cl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol and 2% CHAPS). Lysates were incubated with anti-MCL-1 (Stressgen), anti-Beclin-1 (Santa Cruz) combined with protein-G sepharose (Sigma). Washed beads were processed for western blotting.

For Western blotting, whole-cell extracts were prepared from 1 x 10^6 cells using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton-X-100, 0.1% SDS) supplemented with complete mini protease inhibitor cocktail (Roche Applied Sciences) and 1 mM sodium orthovanadate (Sigma) for 20 minutes on ice. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk and incubated with primary antibodies in 3% bovine serum albumin

(Sigma). Horseradish peroxidase-labeled goat anti-rabbit or anti-mouse antibodies were used for signal detection with chemiluminescence substrate (Pierce) and direct scanning (Fujicolor).

Fluorescence microscopy

ALL cells and MEFs were transfected with the full-length rat pEGFP-LC3 as described above ALL cells were directly co-cultured with MSCs on coverslips for 24h and treated with DMSO, 1 μM DEX, 100 nM obatoclax, or in combination, in the presence or absence of 3-MA (Sigma, 200 μM) for 4 hours. The cells were fixed with methanol for 20 min, washed three times with PBS, mounted with Vectashield on glass slides, and analyzed using a Zeiss META confocal microscope. Mouse embryonic fibroblasts were attached to coverslips for 24 h after transfection and treated with obatoclax or rapamycin for 4 h and then fixed with PFA 4% for 20 min. Nuclei were stained with DAPI in PBS for 10 min. The number of cells with punctate staining per 100 GFP-LC3 transfected cells was determined in two independent experiments. Counting was performed in six random fields, each representing 100 GFP-positive cells and expressed as the mean ± SD.

Transmission electron microscopy

Jurkat cells were treated either with vehicle, dexamethasone and obatoclax (for 72h), TRAIL (for 24h) or with NaAzide (3% for 15min). Cells were harvested, washed with PBS 1X and fixed over night at 4°C in 3% glutaraldehyde in PBS. Cells were then washed and the pellet post-fixed for 1 h at RT with 2% osmium tetroxide and embedded into 2.5% agar in 50 mM sodium cacodylate buffer (pH 7.3), cooled down over night at 4°C and dehydrated in an ethanol series. The samples were diluted with EPON in increasing concentration and embedded in fresh EPON in capsules or flat silicone rubber moulds in the oven at 60°C for at least 12h. The samples were then analyzed using a CM 100 transmission electron microscope.

Viability and Apoptosis Assays

Cell culture was performed in 96-well plates with 2 x 10^4 ALL cells in cell suspension, or as co-culture of 10^5 primary ALL cells on 10^4 MSC, or with 10^5 MEF cells per well. Ten percent of the IC50 concentration of obatoclax or rapamycin was used for combination treatment with DEX, unless stated otherwise. Cell viability for ALL cell lines and MEF experiments was assessed using the MTT cell proliferation kit (Roche Applied Sciences) and, for primary ALL cells, by flow cytometry using 7AAD (BD biosciences). The minimal absorbance of control wells was OD 0.8. Caspase activation was detected by flow cytometry using the CaspGLOW

Red Active Caspase-3 Staining Kit (Alexis) according to the manufacturer's instructions, incubating1 µL specific substrate for caspase-3 (Red-DEVD-FMK) for 10^5 treated cells.

Ratiometric Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential was assessed using the JC-1 mitochondrial membrane sensor (Invitrogen) according to the manufacturer's instructions.

Xenograft model

10^6 primary ALL cells were recovered on dry ice in small portions from cryopreserved presentation samples, washed in PBS, and transplanted intrafemorally into Nod/LtSzScid IL2γ^{null} mice. Xenografted ALL cells were recovered from the spleen and bone marrow, yielding more than 96% human CD45 and C19 positive cells per harvest as verified by flow cytometry, and were cryopreserved for cell culture experiments.
For in vivo treatment, ALL cells recovered from a first xenotransplantation in NSG mice with cells from a precursor B-ALL patient with poor prednisone response were injected intrafemorally into cohorts of NSG mice (10^6 cells per animal). Leukemia progression was assessed by flow cytometry of the mouse peripheral blood using anti-mouse and human CD45 and CD19 antibodies. When 1% human cells were detectable in the peripheral blood, animals were randomized into 4 treatment groups and treated with either vehicle intramuscularly (i.m.; n=11 for patient 4 and n=6 for patient 2), 3 mg/kg/day obatoclax i.m. (n=8 for patient 4 and n=6 for patient 2), 5 µg/g/day DEX intraperitoneally (i.p; n=10 for patient 4 and n=6 for patient 2), or the combination of DEX i.p. and obatoclax i.m. (n=10 for patient 4 and n=6 for patient 2). Animals were treated 5 days per week for three consecutive weeks. Leukemia progression was monitored by flow cytometry.

Supplementary table S1: Cytotoxicity of obatoclax and ABT-737 in ALL cells.
Indicated ALL cell lines were incubated with vehicle, 1 μM DEX, 10% IC_{50} dose of obatoclax, or the combination of DEX and obatoclax for 48h, and caspase activation was measured using flow cytometry. IC50 values obtained from MEF treatment are also presented.

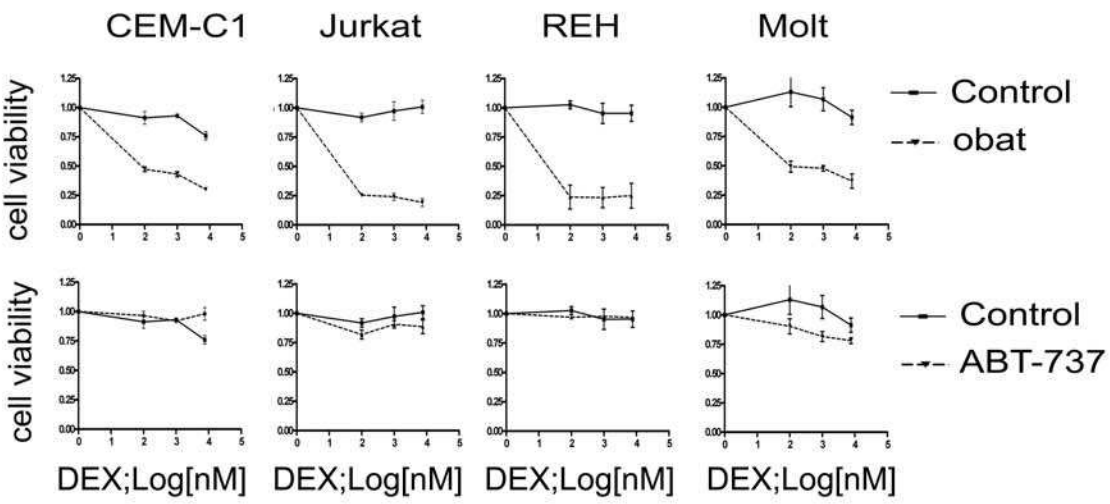
Cell line	IC50 ABT-737, μM	IC50 Obatoclax, μM
CEM-C1	0.242 ± 0.036	0.980 ± 0.145
Jurkat	0.103 ± 0.033	0.500 ± 0.037
REH	1.564 ± 0.13	0.450 ± 0.097
MOLT-4	0.146 ± 0.045	0.055 ± 0.009
CEM-C7	0.058 ± 0.030	0.380 ± 0.079
697	0.182 ± 0.018	0.122 ± 0.023
RS4;11	0.014 ± 0.005	0.113 ± 0.011
wt MEFs	1.234 ± 0.213	0.108 ± 0.025
DKO MEFs	N.D.	0.119 ± 0.022

IC50 values were calculated using GraphPad prism software with triplicate values from MTT assays.

Supplementary Table 2. Patient characteristics

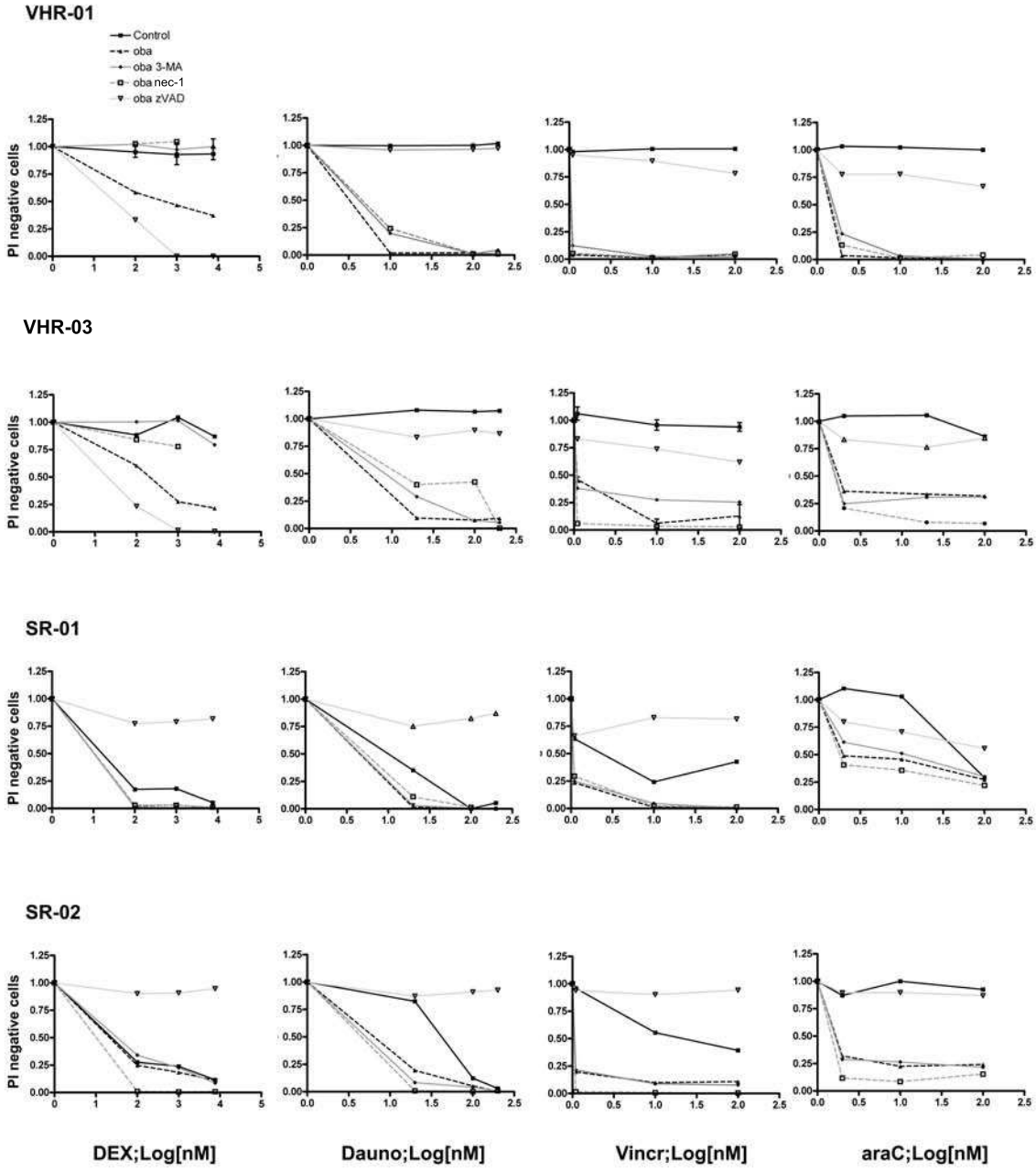
Characteristic	patient no.										
	VHR-01	VHR-02	VHR-03	VHR-05	VHR-06	VHR-07	HR-01	HR-02	SR-01	SR-02	SR-03
Age, y	14	13	17	16	17	12	9	11	11	12	4
Subtype	pc-B	pc-B	pc-B	pc-B	pc-B	pc-B	T	T	pc-B	pc-B	pc-B
Pred.resp.	poor	poor	poor	good	good	poor	poor	poor	good	good	good
Blast, d0	6	80	33	13	16	529	207	75	18	447	11
BM blast d8	1.8	5.7	2.5	0.024	0.164	4.3	127	179	0.017	0.07	0.042
BM blast d0	82%	90%	73%	84%	80%	80%	92%	70%	85%	96%	82%
MRD	VHR	VHR	VHR	VHR	VHR	VHR	HR	HR	SR	SR	SR
Oba IC_{50} (nM)	1284	1521	983	993	1174	1317	1238	1535	923	875	1024

Figure S1



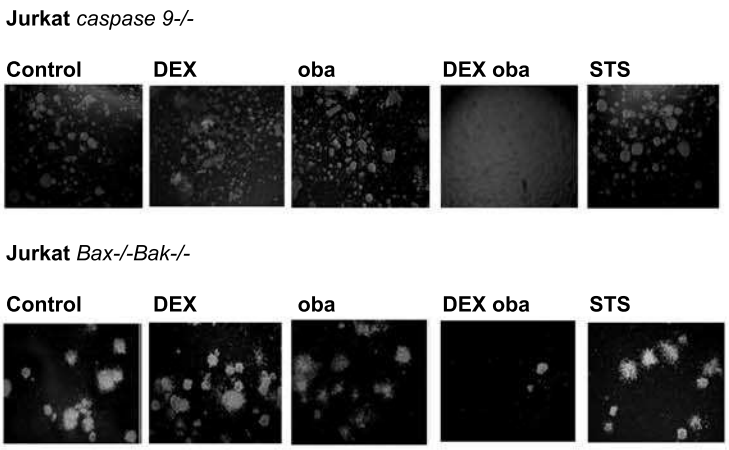
Supplementary Figure S1.
Dose response curves are shown for four different ALL cell lines. Cells were incubated with obatoclax or ABT-737 each at 10% IC50 and increasing concentrations of DEX, and cell survival was assessed with the MTT assay.

Figure S2



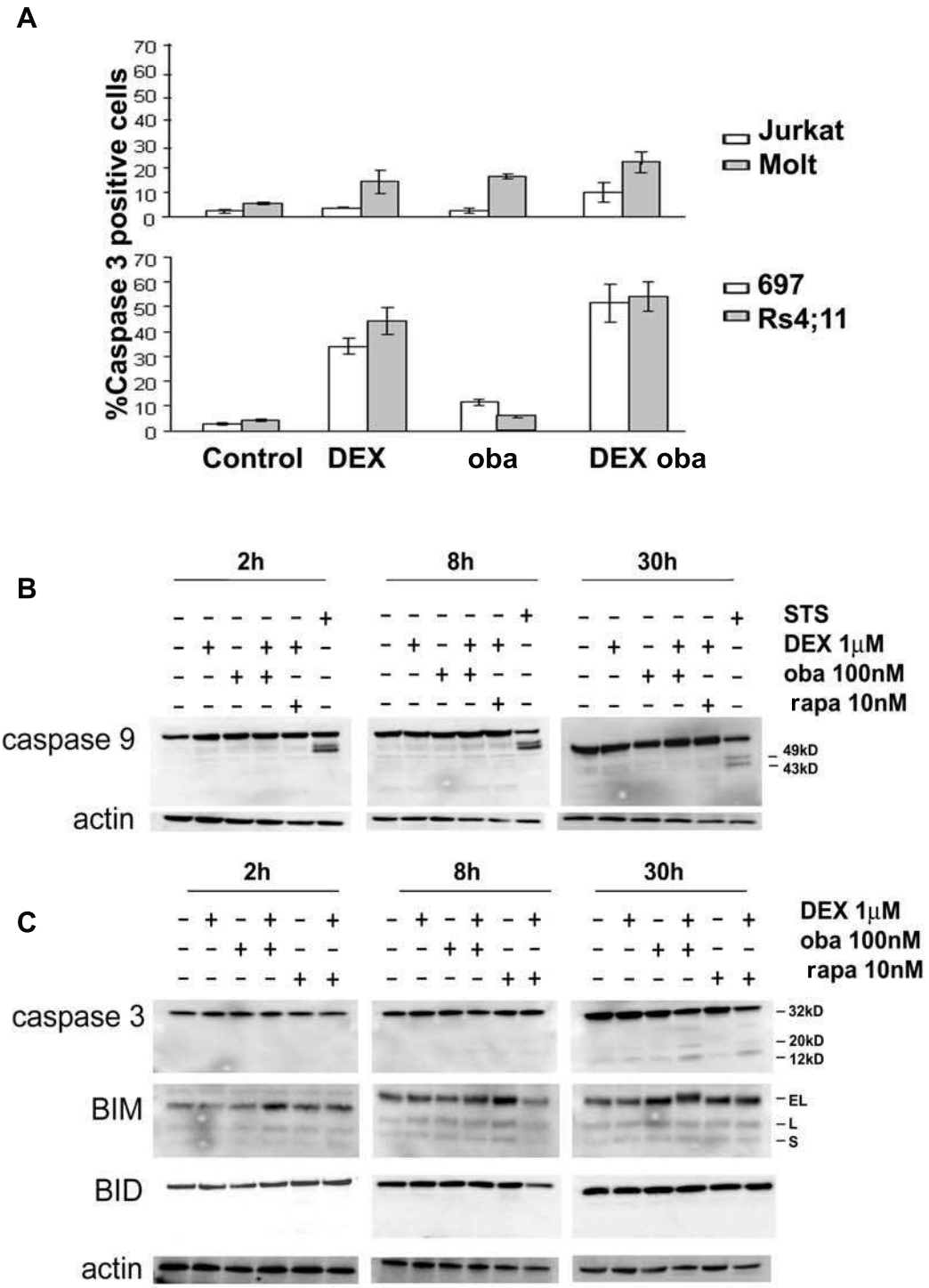
Supplementary Figure S2.
Dose response curves are shown for primary ALL cells from very high-risk (VHR)-ALL and standard-risk (SR)-ALL patients. Cells were cultured on hTERT-immortalized MSCs and incubated for 72h with indicated drugs (obatoclax at 10% IC50, nec-1 at 300 nM, 3-MA at 200 nM and zVAD.fmk at 1 μM) together with increasing concentrations of indicated cytotoxic agents. Cell viability was assessed by 7-AAD stainings using flow cytometry.

Figure S3



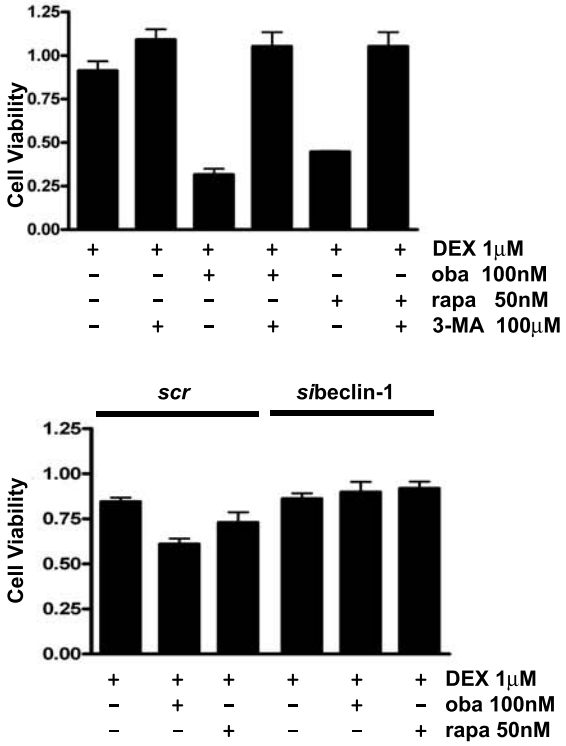
Supplementary Figure S3.
Phase contrast pictures of clonogenic survival assays of Jurkat cells deficient for *caspase-9* (upper panel) or *Bax* and *Bak* (lower panel). Clonogenic survival was assessed after treatment of cells with either vehicle, obatoclax at 100 nM, DEX at 1 μM, their combination or staurosporine at 100 nM for 72h, washing and incubation in methylcellulose for 7 days. Images of representative visual fields, taken on an inverted Leica microscope, are presented.

Figure S4



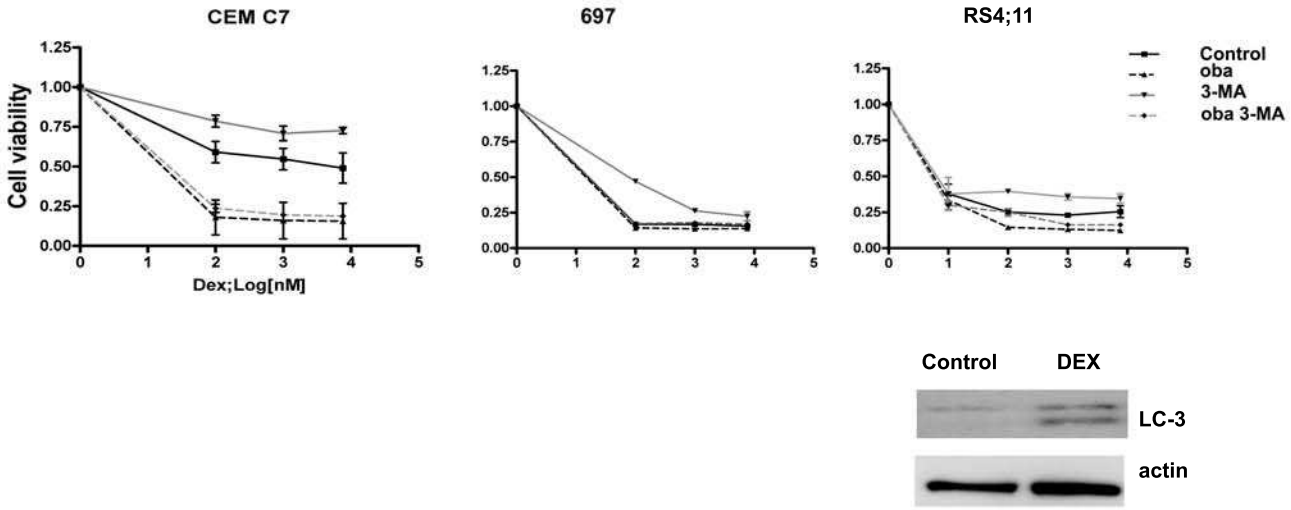
Supplementary Figure S4.
(**A**) Caspase-3 activation in ALL cells was measured by flow cytometry using the CaspGlow Red staining kit. In GC-resistant ALL cells, caspase-3 was only marginally activated after treatment with either dexamethasone, obatoclax or the combination (upper panel). In contrast, in GC-sensitive cells, dexamethasone treatment lead to caspase-activation, which was not further enhanced by co-treatment with obatoclax (lower panel). (**B**) Wild-type Jurkat cells were treated for 2, 8 and 30h as indicated. For control, STS was used. Activation of caspase- 9 was assessed by Western blotting. (**C**) Wild-type Jurkat cells were treated for 2, 8 and 30h as indicated. Activation of caspase-3 as well as levels of BIM and BID were assessed by Western blotting.

Figure S5



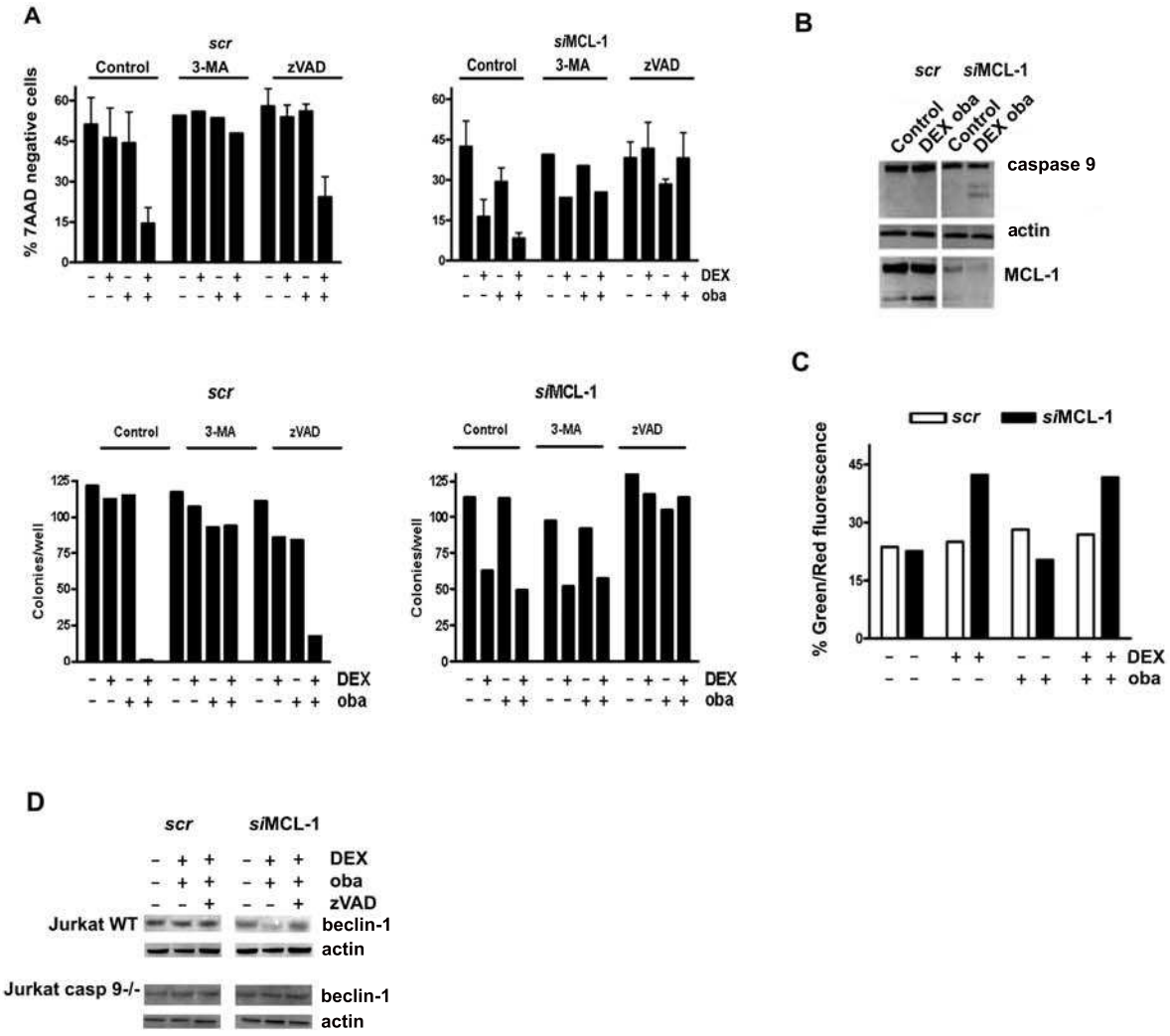
Supplementary Figure S5.
The effect of inhibition of autophagy by either 3-MA or downregulation of Beclin-1 was assessed in an independent cell line, CEM-C1. Cells were incubated for 72h with indicated concentrations of compounds and cell viability was assessed with the MTT assay (upper panel). Likewise, downregulation of beclin-1 conferred resistance to treatment with obatoclax and DEX, as assessed with the MTT assay (lower panel).

Figure S6



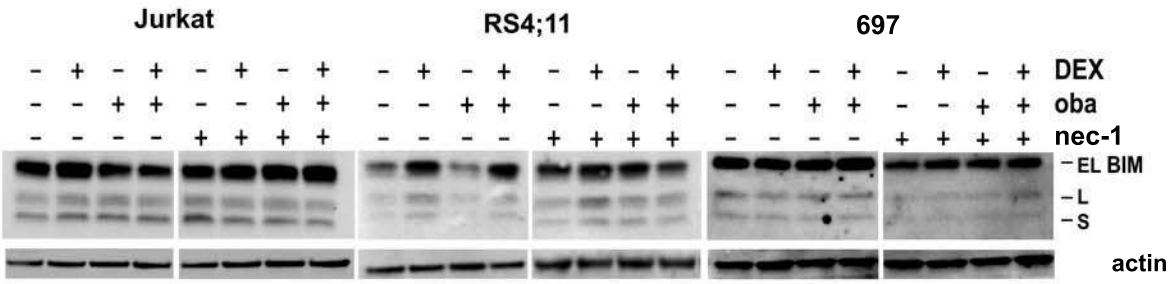
Supplementary Figure S6.
Inhibition of autophagy using 3-MA only partially restored steroid resistance in steroid-sensitive CEM-C7. In the steroid-sensitive lines RS4;11 and 697 cells, no rescue occurred after 3-MA treatment (upper panel). However, LC3-II generation still occurred in RS4;11 cells after treatment with DEX (lower panel).

Figure S7



Supplementary Figure S7. Downregulation of MCL-1 facilitates induction of apoptosis after treatment with DEX.
(**A**) Effect of siRNA-mediated knock-down of MCL-1 on the response to DEX and / or obatoclax was assessed by flow cytometry using 7AAD stainings with or without treatment with indicated agents for 72h. Downregulation of MCL-1 lead to partial sensitization to DEX. (**B**) Activation of caspase-9 after downregulation of MCL-1 and incubation with DEX and obatoclax.
(**C**) Percentage of cells with JC-1 monomers corresponding to cells with a lossof the mitochondrial potential is shown after downregulation of MCL-1 and treatment with vehicle, DEX, obatoclax or the combination. (**D**) Beclin-1 was decreased after downregulation of MCL-1 and treatment with DEX and obatoclax in Jurkat wild type cells (upper panel). Decrease of beclin-1 did not occur in the presence of zVAD.fmk or in Jurkat cells devoid of caspase-9 (lower panel).

Figure S8



Supplementary Figure S8.
Nec-1 inhibits BIM induction in steroid-sensitive ALL cells. Jurkat or RS4;11 cells were incubated with indicated agents for 16h, and BIM induction was assessed by western blotting. In steroid-resistant ALL cells (Jurkat), BIM induction did not occur, and no effect of nec-1 could be observed. In steroid-sensitive RS4;11 cells, BIM induction by dexamethasone was inhibited by nec-1.